UNIVERSITY OF ALBERTA

CARDIOVASCULAR NITRIC OXIDE GENERATION: CHANGES WITH EXERCISE TRAINING AND HEART FAILURE

By

Fadi H. Khadour ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF PHARMACOLOGY

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To my wife, Dina

&

my children, Rammy & Danny

ABSTRACT

Nitric oxide (NO) is a physiological mediator in the cardiovascular system. Changes in NO generation may play a role in cardiovascular adaptations to exercise and heart diseases.

Exercise training is beneficial to cardiovascular function but the mechanisms involved are unknown. In sedentary rats or those trained for 3-4 weeks by swimming, systemic vasopressor and depressor responses to an endothelium-dependent vasoconstrictor (inhibitor of NO synthase, NOS) and vasodilator (acetylcholine), respectively, were augmented by training. A greater reduction in hindquarter blood flow and conductance by the NOS inhibitor and increased responses to both endothelium-dependent and -independent vasodilators were also noted. Training increased Ca^{2+}-dependent NOS activity and expression of endothelial NOS (eNOS) in the lung, aorta and atria but not in ventricles. Expression and activity of neuronal NOS were also enhanced in hindquarter muscles by training.

Abnormalities in cardiac NO formation occur in congestive heart failure (CHF) but the alterations in NOS isoforms and their chamber specificity are unclear. In non-paced dogs, and those subjected to 2 days (no CHF) or 21 days of rapid ventricular pacing (severe CHF), the level of plasma nitrate and nitrite (NO_x^-) was elevated after 7 or 21 days of pacing. In paced animals, Ca^{2+}-dependent activity increased only in the left atria but not right or left ventricles after 21 days of pacing and was accompanied by increased expression of eNOS.

To investigate changes in cardiac NOS during the development of CHF, a genetic model of spontaneously hypertensive and heart failure-prone (SHHF) rats was used, in comparison to spontaneously hypertensive rats (SHR) and

Sprague Dawley (SD) rats at 6, 12 and 18 months of age. Plasma NO_x^- was elevated in SHHF and SHR rats at 18 months only. Increased left ventricular Ca^{2+}-dependent NOS activity and eNOS protein occurred with age in SHHF rats. In SHR rats, the increase in enzyme activity was less over time and occurred without changes in eNOS protein. No changes were observed in SD rats.

These studies establish that increased NO production occurs in both exercise training and CHF. It is speculated that enhanced NO production mediates some of the cardiovascular adaptations to exercise training whereas in CHF this may be of advantage due to the cardioprotective properties of NO.

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LIST OF ABBREVIATIONS

7-NINA	7-nitroindazole
ACh	acetylcholine
ADP	adenosine diphosphate
ANOVA	analysis of variance
BK	bradykinin
BSA	bovine serum albumin
cAMP	cyclic adenosine-3´,5´-monophosphate
cGMP	cyclic guanosine-3´,5´-monophosphate
CHF	congestive heart failure
DNA	deoxyribonucleic acid
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT-4	glucose transporter
HQBF	hindquarter blood flow
HQC	hindquarter conductance
IL-1β	interleukin-1beta
INF-γ	interferon-gamma
iNOS	inducible nitric oxide synthase
L-NAME	N^G-nitro-L-arginine methylester
L-NMMA	N^G-monomethyl-L-arginine
MABP	mean arterial blood pressure
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NE	norepinephrine
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NO_x^-	nitrate and nitrite
$ONOO^-$	peroxynitrite
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein kinase
PKC	calcium-sensitive protein kinase
PKG	cGMP-dependent protein kinase
RT-PCR	reverse transcription polymerase chain reaction
SD	Sprague Dawley
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHHF	spontaneously hypertensive, heart failure-prone
SHR	spontaneously hypertensive rat
SNAP	S-nitroso-N-acetyl-penicillamine
TBS-T	Tris-buffered saline with Tween
TFPI	S-Ethyl-N-[4-(trifluoromethyl) phenyl] isothiourea
TNF-α	tumor necrosis factor-alpha

CHAPTER I

INTRODUCTION

Nitric oxide

Over the past two decades, nitric oxide (NO) has been identified as a novel mediator of diverse biological processes that range from vasoregulation to penile erection, from immune defense to neural signaling (141). Furchgott and Zawadzki in 1980 first noticed that the presence of intact endothelium is required for the vasodilator action of acetylcholine in segments of rabbit aorta and discovered a diffusible factor they called endothelium-derived relaxing factor (56). The identification of endothelium-derived relaxing factor as NO was independently established in 1987 by Palmer and co-workers (152) and by Ignarro and colleagues (87). NO is a free radical gas that functions as a biological messenger in physiological systems. Being a small, reactive, diatomic inorganic molecule with an available unpaired electron, NO is well suited for its role as a biological messenger because of its ability to diffuse readily across biological membranes (66). On the other hand, it has a short half-life of about 6-10 seconds in physiological solutions at 37°C, which reflects the existence of several reactants in the biological milieu that inactivate NO.

Biosynthesis

NO is synthesized from O_2 and one of the terminal guanidino nitrogen atoms of L-arginine with the generation of L-citrulline as a co-product. This oxidation involves an unusual transfer of five electrons and the formation of N-hydroxy-L-arginine as an intermediate. At least three distinct enzymes that catalyze the formation of NO in mammalian cells have been described, the so-called nitric oxide synthases (NOS). They are named after the cell type in which they were originally discovered and numbered in the order in which they were cloned: neuronal or brain NOS (nNOS, bNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). Both nNOS and eNOS are expressed under normal physiological conditions whereas iNOS is expressed a few hours after exposure of a variety of cells to immunological stimuli, a process that requires de novo synthesis of iNOS protein. They are calmodulin dependent (nNOS and eNOS) or calmodulin-containing (iNOS) hemoproteins which require co-factors (FMN, FAD, NADPH, heme and tetrahydrobiopterin) for the oxidation of L-arginine. All three isoforms combine two functionally complementary portions, a carboxyl-terminal reductase domain homologous to cytochrome P-450 reductase and an amino-terminal oxygenase domain containing binding sites for heme, L-arginine and tetrahydrobiopterin, the two portions being connected by a calmodulin-binding domain in the middle. On activation all three isoforms function as homodimers. Under physiological conditions, the supply of L-arginine does not appear to be rate limiting for NO synthesis. Interestingly, if the substrate L-arginine is unavailable in sufficient concentration, the electrons are transferred to oxygen, leading to the formation of superoxide and H_2O_2 (160).

Neuronal nitric oxide synthase

Neuronal NOS is a Ca^{2+}-dependent enzyme, approximately 160-kDa in size that has been found in the brain (21, 22, 103), peripheral nervous system (22, 182), spinal cord (21, 182), pituitary (221), kidney (215), adrenal medulla (150), male sex organ (192), epithelial cells (182) and skeletal muscle cells (144, 105) but not in isolated cardiac myocytes. Furthermore, various smooth muscles throughout the body are innervated by nerves, so called non-adrenergic, non-cholinergic "NANC" nerves which release NO as their neurotransmitter resulting in the relaxation of various smooth muscles such as the gut (25) and erectile tissue of the penis (88). The gene coding for this enzyme is located on human chromosome 12 (126). In skeletal muscle, the use of immunohistochemistry has showed that nNOS is localized in type II (fast twitch) but not type I (slow twitch) muscle fibers, and is particularly localized at the sarcolemma (105) where it appears to concentrate at the motor end plate (113). Neuronal NOS is tightly regulated at the transcriptional level by the synthesis of numerous nNOS mRNA transcripts. At least six distinct molecular species of nNOS proteins are expressed in a tissue-specific and developmentally regulated manner (23). For example, skeletal muscle nNOS (nNOSμ) contains 34 additional amino acids compared with brain NOS, owing to an alternatively spliced segment between exons 16 and 17 (193). This alternative splicing produces proteins that differ in both enzymatic characteristics and structural features. In addition, nNOS is phosphorylated by a variety of protein kinases resulting, at least *in vitro,* in diminished enzyme activity (45). Dephosphorylation of nNOS by calcineurin produces the opposite effect (37). Evidence also indicates that the expression of nNOS can be upregulated in the skeletal muscles of experimental animals by endurance training (10), *in vivo* chronic electrical stimulation (171) and by pregnancy and estradiol therapy (212).

Inducible nitric oxide synthase

In contrast to nNOS and eNOS, iNOS is tightly bound to calmodulin (31) and its activity is thus not affected by the level of intracellular Ca^{2+} but is regulated mainly at the transcriptional level (222). The gene coding iNOS, is located on human chromosome 17 (127). Nearly all cells (including macrophages, endocardial and vascular endothelial cells, neonatal and adult cardiac myocytes, fibroblasts, and vascular smooth muscles to name a few) express this 130-131-kDa enzyme a few hours after exposure to bacterial endotoxin and/or pro-inflammatory cytokines such as interleukin-1β, interleukin-6, tumor necrosis factor-α and interferon-γ (5-7, 28, 222). Induction of iNOS by cytokines in macrophages and other inflammatory cells (high output NO synthesis) and its "constitutive" expression in the bronchial and intestinal mucosae (82) are consistent with a role for this enzyme in natural immunity to infection by viral, bacterial, mycobacterial and parasitic pathogens.

Many factors appear to be involved in the complex regulation of iNOS induction in response to cytokines. iNOS expression is promoted by activation of angiotensin II and α-adrenergic receptors, activation of PKC and increases in cAMP, but is blunted by glucocorticoids, salicylates, osteopontin and the anti-inflammatory cytokine transforming growth factor-β (4). The availability of cofactors and substrate also influence iNOS expression and NO biosynthesis. Cytokine treatment of macrophages, for example, results in the co-induction of iNOS together with the cationic amino acid transporters (both high and low affinity) which increase substrate availability (194) as well as increasing tetrahydrobiopterin levels (7).

Endothelial nitric oxide synthase (eNOS, NOS III)

This isoform has been described (with nNOS) as a "constitutive" Ca^{2+}-dependent enzyme and was first characterized in bovine aortic endothelial cells (159). The human gene encoding eNOS is located on chromosome 7 (127). It is found primarily (85-95%) in the particulate fraction of resting endothelial cells (53) whereas iNOS and nNOS are mainly cytosolic in their intracellular localization. Under physiological conditions, eNOS is expressed in vascular endothelial cells, kidney epithelial cells, hippocampal neurons, skeletal myocytes and cardiac sinoatrial, atrioventricular nodal and ventricular as well as atrial myocytes (1, 5, 45, 74, 105, 141, 184, 185, 187, 205).

Agonist-stimulated (acetylcholine, bradykinin and substance P) NO production by eNOS in endothelial cells is transient and requires an increase in intracellular calcium levels above resting concentrations whereas shear stress-induced increase in NO production is more sustained and independent of intracellular calcium changes and involves phosphorylation of tyrosine and interactions with other molecules in caveolae of the plasmalemma (57). Endothelial cell NO production may be stimulated also by deformations of the cell such as stretching (3, 155). In cardiac myocytes, eNOS is also activated by an increase in beating rate (96) and by hypoxia (101). The expression of eNOS can be upregulated by an increase in shear stress (147, 168, 207), exercise and endurance training (10, 190), pregnancy and estrogens (212). This indicates that endothelial NO is an important mediator of flow-induced vasodilatation since blood flow is enhanced in the hyperdynamic circulation of pregnancy and in the setting of exercise. Conversely, eNOS can be downregulated by cytokines and by an increase in the level of intracellular cAMP (15, 225) suggesting that the expression of iNOS by cytokines and the generation of NO in much higher amounts (nanomolar) than that by eNOS (picomolar) suppresses constitutive NO formation. On the other hand, the regulation of eNOS by intracellular cAMP levels may be important for cardiac myocyte responsiveness in the autonomic control of cardiac chronotropy and inotropy.

Endothelial NOS is synthesized as a 150-kDa protein (15), which undergoes a significant post-translational modification before it is targeted to sarcolemmal caveolae in a process that involves myristoylation (26, 188, 191) and palmitoylation (122, 191) as a 135-kDa protein. eNOS is co-precipitated with caveolins (50), the principle structural proteins of caveolae, and both may cycle between plasma membranes and the Golgi apparatus, where eNOS has been localized in endothelial cells (189). Furthermore, a recent study by Sessa *et al.* showed that agonist and shear stress-stimulated NO production leads to the association of heat-shock protein 90 with eNOS resulting in its activation (58). Although the relationship between the intracellular compartmentalization of eNOS and its enzyme activity in both endothelial and heart muscle cells is not clear at the present time and awaits further study, mutation of *N*-myristoylation site converts eNOS to a cytosolic protein (26, 188). Bradykinin promotes depalmitoylation of eNOS and its translocation to the cytosol (175). Endothelial NOS may also be phosphorylated by PKC, PKA and calmodulin kinase 2 (136).

The regulation of the subcellular localization of eNOS by integrated control of acylation and phosphorylation may have important functional consequences. The presence of eNOS within the caveolae, which interacts with cytoskeletal elements, may facilitate its activation by physical stimuli such as shear stress and stretching. The microdomain of caveolae, rich in other signaling proteins such as G proteins, may be necessary for the acute regulation of eNOS activity by intraluminal agonists. Since NO (or its derivatives) has possible cytotoxic effects, its synthesis near the plasma membrane potentially limits its toxicity and helps to direct NO to the vascular lumen or to smooth muscles. Conversely, translocation of eNOS to the cytosol from caveolae may serve to uncouple or desensitize the enzyme after prolonged agonist stimulation.

Shear stress is the tractive force acting per unit surface area on the luminal side of the endothelial cell as a result of blood flow (114). In contrast to the perpendicular force on the vessel wall (transmural pressure), shear stress acts in the direction of blood flow, parallel to the wall, to create a frictional force at the surface of the endothelium (124). Assuming laminar flow, the magnitude of the shear stress (τ) is proportional to the viscosity (μ) and flow rate (Q) and inversely proportional to the third power of the internal vessel radius (R) according to Poiseuille-Hågen relationship:

$$\tau = 4\mu Q/\pi R^3$$

A number of endothelial cell responses to flow and shear have been described. When exposed to flow, endothelial cell shape changes from polygonal to fusiform (119). These morphologic changes are associated with alignment of the cell major axis in the direction of flow (119). In addition, an increase in shear induces the release of NO (112, 137, 178) and prostacyclin (67) but suppress that of endothelin-1 (125), suggesting their possible involvement in a negative feedback loop of vessel control in response to changes in flow. Shear stress changes the rate of pinocytosis (36) and low-density lipoprotein binding and internalization to endothelial cells (197). It has been implicated also in endothelial cell cycle control and regulation of DNA synthesis (35, 120).

Since 1995 contradictory results concerning the possible presence of NOS in the mitochondria have been published. Kobzik *et al.* found a high Ca^{2+}-dependent NOS activity in the mitochondrial fraction of skeletal muscle homogenates (104). Immunohistochemical studies, using NADPH diaphorase staining (11, 54) and silver enhanced immunolabelling (12) provided evidence for the presence of NOS-like proteins in several locations within the mitochondria. Co-localization of eNOS with succinate dehydrogenase as a mitochondrial marker (104) and cross-reaction of mitochondria with antibodies directed against eNOS (11, 12, 105) and nNOS (54) have been reported. More recent studies suggested the presence of a functional Ca^{2+}-dependent NOS in the mitochondria (mtNOS) with characteristics intermediate between nNOS, eNOS and iNOS (63, 203) and that mitochondria are a source of NO that may affect energy metabolism, O_2 consumption and free radical formation (65).

Mechanism of action, interactions and metabolism

Once produced, NO rapidly diffuses across the cell membrane to interact with specific molecular targets, but the biological effects of NO are likely to be modulated in different microenvironments by: 1) the rate and/or duration of NO production (i.e. NO synthesis by the high output iNOS tends to be greater and more prolonged than by eNOS), 2) the number and the variety of available molecular targets within diffusion distance from the source of NO synthesis along with their relative reaction rates with NO, and 3) the net redox state that prevails in the cell. A predominant targets of NO are the heme-containing proteins, particularly the heme moiety of soluble guanylyl cyclase. NO induces a conformational change that displaces iron out of the plane of the porphyrin ring (86), resulting in the activation of guanylyl cyclase and increased synthesis of cyclic guanosine-3',5'-monophosphate (cGMP). NO interacts with heme moieties in oxyhemoglobin and myoglobin resulting in the inactive metabolite nitrate (109, 145, 213). Another interaction of NO with the heme of cyclooxygenase 2 enhances the formation of prostaglandins (180). Because nitrate is a stable metabolite of NO in man and animals, plasma nitrate has been used as an index of the endogenous formation of NO. In aqueous solution, NO is rapidly oxidized to nitrite (152), which is itself oxidized *in vivo* to nitrate (220).

Other major targets of NO reactivity are reduced thiol residues on most intracellular, membrane-associated, or plasma proteins (albumin) or free thiols such as glutathione and cysteine (198). This result in the nitrosylation of these targets which may affect their biological function either positively or negatively. By reacting with glutathione to form *S*-nitrosoglutathione, NO can deplete intracellular stores of reduced glutathione resulting in activation of the hexose monophosphate shunt (32). NO stimulates the auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in inhibition of glycolysis (44). NO can also directly inhibit NADPH oxidase (33).

NO binds and inactivates iron-sulfur complexes in enzymes such as *cis*-aconitase (46), NADH succinate oxidoreductase and NADH ubiquinone oxidoreductase (mitochondrial complexes I & II) (62), and cytochrome *c* oxidase (complex IV) (24). In addition, NO has been shown to reduce oxygen consumption in isolated bovine cardiac muscle slices, presumably by inhibiting mitochondrial electron transfer (224). Similarly, reduced oxygen consumption was reported in isolated segments of canine myocardium incubated with NO donor drugs or agonists which stimulate endogenous NO release such as bradykinin or carbachol, (223). These results suggest that NO controls cardiac mitochondrial respiration and perhaps this is a mechanism by which it may act as a physiological regulator of cardiac function. Therefore, changes in cardiac NO biosynthesis may be responsible for myocardial dysfunction in pathological conditions such as heart failure and ischemia-reperfusion injury.

Large amounts of NO can also promote DNA strand breaks (146), which can initiate apoptosis or activate poly-ADP ribose synthetase (226), leading to depletion of cell energy stores and death. NO can also inhibit DNA synthesis by binding the non-heme iron of ribonucleotide reductase (118).

More recent studies have indicated that many of the deleterious effects of NO, including its interactions with some of the above mentioned enzymes and proteins, are mediated by peroxynitrite ($ONOO^-$), a more powerful and toxic oxidant formed by the reaction of NO with superoxide (13). Peroxynitrite has been reported to inhibit mitochondrial aconitase (27, 77), complexes I and II of the mitochondrial electron transport chain (162) cytochrome c reductase and cytochrome oxidase (18), and mitochondrial ATPase (162). It also causes lipid peroxidation (179), oxidation of sulfhydryl moieties (161) and DNA strand breakage (99, 227) which can activate poly-ADP ribose synthetase thus depleting the intracellular stores of its substrate NAD^+. In addition, it can directly nitrate free and protein-associated tyrosine residues (14).

The different NO metabolites and adducts that appear from interaction between NO and various intracellular and extracellular targets may represent pools of biologically inactive NO (i.e. nitrate) or a form of long term storage for

NO (i.e. nitrosothiols) and thus serve to buffer NO effects when produced but also may prolong its action after its initial biosynthesis.

Nitric oxide in the cardiovascular system

Effects on blood vessels

NO is continuously produced by vascular endothelial cells, and this basal release of NO is a major determinant of vasodilator tone (68, 141), blood pressure and blood flow, both in large arteries and at the microcirculatory level (59, 170, 209). On the other hand, blood flow is probably the most important physiological stimulus for endothelial NO generation and the highest NO activity is found in larger diameter arteries that are subject to greater variation in pulsatile flow and shear stress (68, 112, 137, 178). The release of NO from the coronary endothelium is also involved in the regulation of the coronary circulation (1, 97). NO also inhibits migration and proliferation of vascular smooth muscle cells *in vitro* (60, 181). Moreover, NO is intimately involved in maintaining the integrity of the vascular endothelium by modulating platelet-vessel interactions.

Effects on platelets

There is substantial *in vitro* evidence that NO is a potent inhibitor of platelet function. NO inhibits ADP-induced platelet aggregation and also disaggregates platelets, via a cGMP-dependent mechanism (134, 167). It has a synergistic effect with prostacyclin to inhibit platelet aggregation (123, 167). Furthermore, platelets themselves contain NOS and they generate NO when stimulated to aggregate (163, 164). This response acts as a negative feedback mechanism to inhibit platelet activation. NO also inhibits the adhesion of platelets (165, 166) and leukocytes (110) to the vascular endothelium and the induction in endothelial cells of leukocyte adhesion molecule (VCAM)-1 by cytokines (38).

Effects on myocardial inotropy and lusitropy

There is now convincing evidence that NO exerts physiologically important and direct influences on myocardial contractility. The first study to demonstrate a role for NO in the regulation of myocyte function was performed in isolated ferret papillary muscles whereby endothelium-derived relaxing factor and NO donors were found to induce an earlier relaxation and therefore shorten the duration of contraction without significantly altering the peak or rate of force development (196). Subsequently, other investigators also reported a negative inotropic effect for NO in isolated cardiac myocytes (20, 202) and on the heart (51, 69, 70). Studies in normal human subjects have yielded similar results with enhanced relaxation and reduced diastolic tone of the left ventricle (153, 154). Excess NO from the induction of iNOS expression in isolated ventricular myocytes (6, 7, 19, 184) or working hearts (183) has also been found to depress myocardial contractile function, an effect that can be blocked by inhibitors of NO synthesis. Therefore, both cardiac eNOS and iNOS generate enough NO to exert a negative inotropic action.

Since cardiac myocytes are in close proximity to coronary vascular (≤ 8 μm) (176) or endocardial endothelia and the maximal diffusion distance of NO is 200-600 μm (102), NO released from these cells also influences cardiac function. Bradykinin-stimulated NO production from endothelial cells co-cultured with guinea pig cardiac myocytes reduced the contractility of myocytes, an effect that was reversed with NOS inhibitors, hemoglobin (a NO scavengers) and an inhibitor of soluble guanylyl cyclase, methylene blue (20). Additional evidence for paracrine effects of microvascular endothelial cell-derived NO on adjacent myocardial cells was provided by studies in ferret papillary muscle (196) and in isolated guinea pig hearts (69), whereby endogenous NO accelerated diastolic ventricular relaxation without significant effects on the rate of systolic development. Activation of iNOS in endothelial cells by cytokines can depress the contractile responsiveness of the adjacent myocytes (208).

NO also appears to play an important role in the autonomic modulation of myocardial function. Balligand and colleagues have shown that an endogenous NO signaling pathway modulates the responsiveness of rat myocytes to adrenergic stimuli (9). They showed that inhibition of NOS in cultured adult rat ventricular myocytes significantly augmented the positive inotropic response to isoproterenol, but had no or little effect on the basal contractile state. A recent report by Gauthier *et al.* found that the negative inotropic effect of β_3-adrenergic receptor stimulation is mediated via NO in human ventricle (61). Furthermore, synthesis of NO by cytokine-induced iNOS can inhibit the β_1–adrenergic pathway-mediated increase in myocyte contractility (6, 7, 9, 90). Therefore, both basal and cytokine-induced NO can regulate the myocardial inotropic response to β-adrenergic stimulation. There is also limited evidence to suggest that NO modulates the positive inotropic effect of the α-adrenergic pathway in ventricular myocardium (49). In addition, NO plays a significant role in mediating the effect of muscarinic receptor agonists to inhibit the positive inotropic response to β–adrenergic stimulation (8).

Effects on myocardial chronotropy

Roberts *et al.* have shown that exposure to pro-inflammatory cytokines suppresses the spontaneous beating rate of cultured cardiac myocytes from neonatal rats by a NO-dependent mechanism (174). There is also indirect evidence for inhibition of the positive chronotropic effect of β–adrenergic stimulation by NO (71). Additional experiments, using spontaneously beating cardiac myocytes from the neonatal rat heart, showed that inhibition of NO by L-NMMA prevented the negative chronotropic effect of muscarinic receptor stimulation (9). However, NOS inhibitors do not accelerate the spontaneous beating rate in cultured neonatal myocytes (9) or when administered via the coronary artery to humans (75). Therefore, it appears that NO is also involved in the autonomic regulation of heart rate.

Other effects of NO

Excess NO production by iNOS may diminish cardiac function by increasing cell death, possibly by triggering apoptosis. Pinsky and co-workers found that induction of iNOS by pro-inflammatory cytokines in isolated adult rat cardiac myocytes was associated with a NO-dependent cytolytic effect (157). Other studies have also indicated that NO donors can trigger apoptosis in cardiac myocytes (149, 158), macrophages, and in vascular smooth muscle.

Mechanisms of cardiovascular effects of NO

The effects of NO in the cardiovascular system are mediated in most but not all instances via elevation of cGMP. This is fundamental in processes such as endothelial regulation of vascular smooth muscle tone (141) and proliferation (60) as well as inhibition of platelet adhesion and aggregation (134, 166). On the other hand, the molecular mechanisms by which NO influence myocardial performance are subjects of extensive investigation but remain unclear. Much attention has been given to cGMP-activated pathways with the main target proteins for cGMP in the myocardium believed to be cGMP-dependent protein kinase (PKG) and phosphodiesterases. Multiple effects have been reported upon the activation of PKG, including a reduction in calcium current intensity (135) and desensitization of cardiac myofilaments for calcium (156). NO or cGMP may also decrease cardiac myocyte L-type calcium current and contraction through activation of cGMP-stimulated cAMP phosphodiestease, resulting in a decrease in intracellular cAMP levels and the activity of PKA which alters the phosphorylation of several target proteins (76). Other mechanisms independent from cGMP elevation may operate through interaction of NO and/or $ONOO^-$ with heme proteins, non-heme iron, or free thiol residues on target signaling proteins, enzymes, or ion channels.

Nitric oxide and cardiovascular adaptations to exercise

Cardiac and hemodynamic changes in acute exercise

An acute bout of exercise challenges the cardiovascular system to increase delivery of O_2 and other metabolic substrates to the actively contracting muscles as well as enhance the removal of metabolic by-products. This is achieved by an impressive increase in cardiac output along with redistribution of blood flow resulting in a dramatic increase in the perfusion of cardiac muscle and exercising skeletal muscles (129). In humans, cardiac output may double or even triple during aerobic exercise from the normal value of 6-8 L/min. This increase in cardiac output is mainly related to increased heart rate but also greater stroke volume due to increased preload (venous return) and cardiac contractility. In addition, total peripheral resistance decreases as a result of exercise-induced metabolic vasodilatation of skeletal muscle arterioles resulting in decreased diastolic blood pressure. In contrast, systolic blood pressure increases because of increased cardiac output. Simultaneously, blood flow to the skin, kidneys, splanchnic organs and non-exercising skeletal muscle decreases significantly (128). This redistribution is achieved via vasoconstriction in relatively inactive organs and dilatation of the vasculature serving active skeletal muscles and the heart (129).

Cardiac and hemodynamic adaptations to exercise training

Repeatedly challenging the cardiovascular system to respond to the increased demand for O_2 and nutrients results in a number of physiological changes as part of the body's adaptation to chronic exercise. In trained athletes and experimental animals, the resting heart rate decreases as the balance between sympathetic and parasympathetic neural stimulation of the heart is altered. A physiological cardiac hypertrophy also occurs with training. In fact, an increase in total heart weight and specifically ventricular weight is commonly used in animals to confirm the efficacy of the training regimen (100, 201). Previous studies have also reported a distinct increase in atrial weight of trained animals (100). Together with increased citrate synthase activity in skeletal muscles, a marker of mitochondrial content, these are important signs of endurance training in human subjects and experimental animals.

Evidence indicates that the endothelium mediates and/or participates in vasodilator and constrictor responses as well as the structural vascular adaptation in response to exercise training. Several reports have documented a diminished aortic vascular reactivity to various vasoconstrictors in experimental animals exercised by running (29, 30) or swimming (89). Studies have also shown the enhancement of endothelium-dependent, acetylcholine-mediated dilatation of the abdominal aorta in exercise trained rats (42, 43). This is because of the ability of the endothelium to sense physical forces imparted to the blood vessel wall (shear stress and stretching) as well as chemical substances within the blood and to initiate responses to these stimuli by releasing NO and other substances that modulate vascular tone and vessel structure.

A previous study by Sessa *et al.* reported enhanced expression of eNOS mRNA in aortic endothelial cells of dogs by short-term exercise (running for 1 h, twice daily for 10 days) (190). In addition, Delp *et al.* found increased eNOS protein expression in the abdominal aortas of rats exercised by running for 4 weeks (42).

Skeletal muscle adaptations to exercise training

Although skeletal muscle perfusion is low at rest, it increases during exercise in proportion to the exercise intensity. Muscle perfusion during exercise is regulated through central and local vascular control mechanisms (41). Local vascular control mechanisms include metabolic control, endothelium-mediated control, propagated control, myogenic control and the muscle pump. The primary determinant of muscle perfusion during exercise is the metabolic rate of the muscle. Metabolites from contracting muscle diffuse to resistance arterioles and act directly to induce vasodilatation or indirectly to inhibit norepinephrine release from sympathetic nerve endings (41). Myogenic autoregulation does not appear to be involved in exercise hyperemia in muscle but the rhythmic propulsion of blood from skeletal muscle veins (the muscle pump) facilitate venous return to the heart and thus muscle perfusion (41). In addition, signals released from the vascular endothelium also contribute to the hyperemic response of contracting skeletal muscle. At least two such endothelium-derived vasodilator substances that may be involved in the regulation of blood flow to skeletal muscle have been established: prostacyclin and NO (107). In fact, data suggests that endothelium-dependent, flow-induced vasodilatation is an integral mechanism in the regulation of vascular tone in skeletal muscle that is mediated at least in part by NO (80).

Experimental evidence using the radiolabeled microspheres technique has demonstrated an enhancement of blood flow in the hindquarter skeletal muscles of exercised animals (83, 117, 142). Hirai and co-workers in 1994 reported that endothelium-derived NO contributes to exercise-induced dilatory response in hindlimb muscles in exercised rats (moderate intensity treadmill running) (80). In isolated skeletal muscle arterioles, the release of both NO and prostaglandins was upregulated by short-term daily exercise, and inhibition of synthesis of either of these substances reduced flow-mediated dilatation of the arterioles to a similar degree (40-45%) whereas combined administration of the inhibitors nearly abolished the dilatation of the arterioles (107, 201).

Whether the action of NO is important during exercise-induced hyperemia in human skeletal muscles is equivocal. Gilligan *et al.* and Katz *et al.* reported that intrabrachial artery infusion of the NOS inhibitor L-NMMA reduces handgrip exercise-induced vasodilatation in the human forearm (64, 95). Hindquarter skeletal muscle blood flow, measured by microdialysis probes in the quadriceps femoris of healthy subjects, was also reduced both at rest and during continuous dynamic exercise by L-NMMA (79). On the other hand, Wilson and Kapoor and Endo *et al.* found that regional inhibition of NOS has either no or minimal effects on the hyperemic blood flow response to forearm exercise (48, 216). Even the systemic administration of L-NMMA at a dose (3 mg/kg) that induced moderate hemodynamic changes did not affect exercise-induced vasodilatation to the skeletal muscle (108).

Visceral perfusion in exercise training

Reduced splanchnic and renal blood flows during acute dynamic exercise are characteristic of many species, including humans (177), baboons (83), rabbits (128) and rats (80). The magnitude of reduction in splanchnic and renal blood flows appears to be directly related to the relative exercise intensity in most species. Increased sympathetic nervous system outflow to the vasculature of the splanchnic and renal circulations is the primary factor responsible for reduced flow to these areas during acute exercise (128). However, angiotensin II (199) and vasopressin (200) have been shown to contribute to the redistribution of the cardiac output during acute exercise. In addition, the study by Hirai *et al.* demonstrated that in rats exercised by running on a treadmill, the administration of a NOS inhibitor during exercise reduced blood flow to the abdominal organs (kidneys, liver, pancreas, stomach and intestine) suggesting that NO is responsible at least in part for blood flow to these organs (80). Moreover, NO also plays a role in regulating blood flow through the mesenteric arterial bed (94, 143).

Plasma NO_x^- and exercise training

Plasma nitrate, a stable by-product of endogenous NO metabolism, has been used by a number of investigators as an index of NO generation in the setting of acute exercise and training. In humans, Jungersten *et al.* found that the resting plasma nitrate level is higher in athletic subjects than non-athletic controls and acute exercise (135 min of jogging a distance of ~25 km or 120 min of bicycling) significantly elevated plasma nitrate levels in both groups (92). In spontaneously hypertensive rats, a biphasic change in plasma nitrate level was reported with chronic voluntary exercise (91). During spontaneous running in exercise wheels over a period of 3-35 days, nitrate levels decreased after 7 days then returned to approximately pre-training values at 14 and 21 days and were significantly increased above pre-training level after 35 days of exercise

(91). Cardiac NO production, measured as the difference between aortic and coronary sinus NO_x^- levels, was also increased in dogs exercised by running on a treadmill for 5 min at different speeds (16).

Nitric oxide and congestive heart failure

Congestive heart failure (CHF) is a complex syndrome of impaired myocardial function and decreased cardiac output. As a compensatory mechanism there is activation of various neurohumoral pathways in proportion to the severity of heart failure, resulting in a state of peripheral vasoconstriction and fluid retention. At least two neurohumoral mechanisms have been found to participate in the vasoconstriction: the sympathetic nervous system and the renin-angiotensin system. CHF is a difficult problem to investigate because of its variable etiology, uncertainty in defining its time of onset, and the multiple cardiac and peripheral alterations that occur. Therefore, despite numerous studies to date, the exact pathogenesis of this syndrome remains unknown.

Animal models of heart failure

There are several experimental models of CHF that simulate many features of the clinical syndrome in human patients. Animal models of CHF can be classified into six categories according to the type of inducing stimulus:

a) Pressure overload. This may be applied to the right ventricle (pulmonary artery banding or pulmonary valve stenosis) or the left ventricle (aortic constriction or aortic valve stenosis) to produce right or left ventricular hypertrophy, respectively, and thence heart failure (85). In this model, changes in the heart develop over several weeks and are, therefore, more acute that those seen in human patients.

b) Volume overload. Acute heart failure (within hours) has been induced by intravenous infusion of fluids to anesthetized baboons (172). On the other hand, surgical techniques involving the creation of aorto-vena caval fistulae, incompetence of the aortic or pulmonary valves or atrial septal defects can reliably induce patterns of CHF within weeks that mimic precisely the clinical situation (52).

c) Myocardial infarction and destruction. Myocardial infarction and failure may be produced by ligation or embolization of a coronary artery (151). Cardiac failure has been produced also by chronic hypoxia and repeated electric shock to the heart.

d) Chemically-induced cardiomyopathy. Myocardial disease may also be induced by a variety of chemicals, such as barbiturates, adriamycin and catecholamines administered acutely (98) or chronically (115).

e) Tachycardia-induced heart failure. Another technique for the induction of CHF is by rapid pacing of the heart.

f) Spontaneous cardiomyopathy may occur in certain animal species (Syrian hamsters, turkeys and spontaneously hypertensive heart failure-prone, SHHF, rats) resulting in CHF.

The last two models were employed in this thesis, therefore I will expand upon them in the sections which follow.

Pacing (tachycardia)-induced heart failure

In 1962, Whipple *et al.* reported that atrial pacing in dogs induced CHF (214). Subsequently, right ventricular pacing was employed by Armstrong and others and has been extensively characterized (2, 138-140, 173, 186, 217). Rapid atrial or ventricular pacing is currently a widely used method of inducing heart failure in several animal species. In the dog, chronic asynchronous right ventricular pacing at rates exceeding 200 beats/min can induce severe heart failure within 2-8 weeks with clinical, radiographic, echocardiographic, hemodynamic and neurohumoral alterations very similar to those observed in humans (2, 85, 138-140, 173, 217). This model offers certain advantages in that: *a*) it avoids major surgical trauma, *b*) the pacing rate can be controlled by an external programmable pacemaker and *c*) CHF occurs in a predictable time course. A unique finding in this canine model of CHF is the development of atrial (138, 186) but not ventricular hypertrophy despite intense hemodynamic stress and neurohumoral stimulation [2, 138-140, 173, 186, 214).

SHHF rats

The SHHF/Mcc-fa^{cp} (abbreviated as SHHF) rat model is a relatively new genetic model of non-insulin-dependent diabetes mellitus, obesity, hypertension, and heart failure (132). These animals were developed by backcrossing Koletsky obese rats to spontaneously hypertensive rats (106). All of these rats are hypertensive and eventually develop spontaneously dilated cardiomyopathy and CHF (133). SHHF rats have systolic blood pressure ranging from 145 to 210 mmHg, a range similar to that observed in SHR rats, but with the onset of severe CHF the blood pressure falls to normotensive levels (110-130 mmHg) (133). These rats initially show hypertrophy of the left ventricle, followed by right ventricular hypertrophy and eventually hypertrophy and dilatation of the atria. Terminally there is dilatation of all four chambers of the heart (133). Ventricular myocyte hypertrophy is gradually but progressively replaced by interstitial fibrosis, mostly pronounced in the endomyocardial areas of both ventricles (130), as animals progress into CHF. The age of expression of overt symptoms of CHF is very dependent on both the genotype and gender in SHHF rats. Obese male rats develop CHF at 10-14 months of age, obese female at 14-18 months, lean males at 14-25 months, and lean females at 19-31 months (13). When in failure, these rats exhibit the clinical signs of CHF together with hemodynamic changes and both electrocardiographic and echocardiographic evidence characteristic of CHF (130, 72). Moreover, CHF in SHHF rats is associated with the elevation of circulating neurohumoral factors such as norepinephrine, atrial natriuretic peptide, vasopressin, aldosterone and plasma renin activity similar to that seen in man (84, 130, 131).

Peripheral and coronary vascular NO in CHF

The increase in vascular resistance in heart failure is only partly explained by known neurogenic and humoral stimuli. Structural changes and failure of an effective counter-regulatory vasodilator system may be involved. In a study of heart failure patients Winlaw *et al.* observed that plasma levels of nitrate were elevated in these patients compared with healthy individuals (218). Subsequently, the same investigators reported that the increase in plasma nitrate levels positively correlated with the severity of heart failure (219). These data suggest an increase in NO production in CHF, the source of NO may be the vascular endothelium, although the actual site was not identified. Nevertheless, it has been suggested that the increase in plasma NO_x^- during CHF is due to a decline in renal function and, therefore, reduced urinary excretion of NO_x^- and not an increase in NO production (17). This is based on the finding of Bernstein *et al.* who reported in dogs paced for 4 weeks into CHF (210 beats/min in the first 3 weeks and 240 beats/min for another week) no changes in plasma NO_x^- levels within the first 3 weeks of pacing, whereas they rose in the fourth week in direct proportion to the increase in plasma creatinine levels. More recent data from the same investigators in the same experimental model indicated that cardiac NO production (the difference in plasma NO_x^- levels between aortic and coronary sinus blood) falls below normal levels after the development of heart failure (169).

Studies of peripheral vascular and coronary endothelial function in relation to NO production in CHF have revealed conflicting data. A reduction in endothelium-dependent relaxation in response to acetylcholine in the femoral artery of dogs (93) and the hindquarter circulation of rats with CHF (47) has been reported. These findings are supported by data showing diminished eNOS mRNA and protein expression in the aortic endothelium of dogs (195) and in rat aorta (34) in heart failure. Similarly, in patients with heart failure, measurement of forearm blood flow confirmed the blunting of acetylcholine-induced forearm vasodilatation (81, 111, 206) but endothelium-dependent vasorelaxation induced by substance P was unaffected (81). On the other hand, O'Murchu *et al.* found no difference in the responses of isolated femoral arteries from dogs with CHF induced by rapid atrial pacing to a selective α_2 receptor agonist and endothelium-dependent vasodilator (BHT 920) (148).

In the coronary circulation, enhancement of endothelium-dependent relaxation responses in the coronary arteries of dogs with pacing-induced CHF to BHT 920 and acetylcholine has been reported (116, 148). Conversely, Wang *et al.* reported a diminished coronary blood flow response to acetylcholine in the same model of CHF and also found that large coronary arteries and microvessels from canine failing hearts produced less nitrite *in vitro* than those of normal hearts in response to the same vasodilator (211). These findings suggest that agonist-induced endothelium-dependent relaxations may be affected heterogeneously in heart failure depending on the vasodilator and the vascular bed examined.

Myocardial NOS in CHF

There is now considerable evidence that NO production is altered in the failing myocardium, however, the cause(s) of this alteration and the role of NO in the pathogenesis of heart failure are still to be clarified. DeBelder *et al.* were the first to investigate changes in Ca^{2+}-dependent and -independent NOS activities in the human failing myocardium. They found a significant Ca^{2+}-independent NOS activity in right ventricular myocardial biopsy specimens of patients with dilated cardiomyopathy, inflammatory cardiomyopathy and myocarditis (39, 40) whereas Ca^{2+}-dependent NOS activity was detected in both dilated and non-dilated hearts of patients with ischemic and valvular disease (39). Expression of iNOS mRNA and protein in the ventricles in end-stage heart failure resulting from ischemic and valvular heart diseases, as well as dilated cardiomyopathy was reported by Haywood and co-workers (78). Moreover, Habib *et al.* found that expression of iNOS protein in the myocardium of patients with dilated cardiomyopathy co-localized with that of TNF-α (73), a cytokine which induces the expression of iNOS in various cells including cardiac myocytes (184) and whose circulating plasma levels are known to increase in CHF (121). In contrast, Thoenes *et al.* detected iNOS protein in the left ventricular myocardium of failing hearts from patients with septic shock but not in heart failure due to other causes or in non-failing human hearts (204).

More recently data from two separate groups on the expression of iNOS in cardiac myocytes from failing human hearts have consistently detected both iNOS protein and mRNA by RT-PCR, immunohistochemistry and *in situ* hybridization in the myocardium regardless of the underlying etiology of heart failure (55, 210). However, there is also evidence for increased expression of eNOS protein and mRNA by immunohistochemistry and *in situ* hybridization, though variably, in the subendocardial areas of failing human hearts (55).

Whether excess NO production in heart failure is cardioprotective through its coronary vasodilatory, platelet antiaggregatory and metabolic modulatory actions or detrimental because of its negative inotropic, cytolytic and apoptotic effects is unknown.

OBJECTIVES AND HYPOTHESES

The alterations in cardiovascular NO generation were investigated under both physiological conditions, i.e. adaptations to swim exercise training, and in a pathophysiological setting of CHF using different experimental animal models.

a) Cardiovascular NO generation in rats trained by swimming (Chapter II)

The mechanism(s) of the beneficial effects of exercise on the cardiovascular system, including the changes in vascular endothelial function, has been investigated in various experimental animals and in humans. However, the changes in systemic vascular reactivity and in cardiopulmonary NOS activity and expression induced by exercise training are unknown. In this study, the effect of swim exercise training on systemic vascular reactivity was investigated *in vivo*, along with measurements of the changes in plasma NO_x^- level. In addition, the alterations in Ca^{2+}-dependent and -independent NOS activities and protein expression in the lung, aorta and heart were determined. The following hypotheses were tested:

I. Exercise training enhances systemic vascular endothelial function and NO-mediated responses.

II. Exercise training upregulates Ca^{2+}-dependent NOS activity and eNOS protein expression in organs with increased blood flow during exercise.

III. Resting plasma NO_x^- level is elevated in trained animals.

b) Skeletal muscle and visceral NO generation in swim-trained rats (Chapter III)

During acute exercise, blood flow to the actively working skeletal muscles is increased whereas splanchnic and renal perfusions are reduced. However, the alterations in skeletal muscle and visceral NOS activity and expression, along with changes in hindquarter and mesenteric vasoreactivity caused by exercise training have not been investigated. In this study, the changes in rat's hindquarter vascular reactivity and those in the superior mesenteric bed vasoreactivity induced by swim exercise training were investigated. The alterations in Ca^{2+}-dependent and -independent NOS activities and protein expression in the skeletal muscle, liver and kidney caused by training were also evaluated. The following hypotheses were tested:

I. Exercise training enhances hindquarter vascular endothelial function and NO-mediated responses.
II. Exercise training upregulates Ca^{2+}-dependent NOS activity and eNOS protein expression in skeletal muscle.
III. Superior mesenteric bed vasoreactivity and NOS activity in visceral organs are not altered by exercise training.

c) Cardiac NO production in dogs with pacing-induced CHF (Chapter IV)

In humans, several studies have reported increased expression of iNOS protein and/or mRNA in the myocardium of left or right ventricles of failing hearts but no study has determined the activity and expression of both eNOS and iNOS in unison in the failing heart. Moreover, the cardiac chamber specificity of changes in NOS is unknown. This study determined the alterations in the activity and expression of both eNOS and iNOS in the hearts of dogs with rapid ventricular pacing-induced CHF as well as the chamber specificity for changes in cardiac NOS, and how this may be reflected on the level of plasma NO_x^-. The following hypotheses were tested:

I. Ventricular NO production is enhanced, via iNOS expression, in dogs with pacing-induced CHF.

II. Ventricular iNOS expression is reflected by an increased plasma NO_x^- level.

d) Ventricular NO production in a genetic rat model of heart failure (Chapter V)

Previous studies provided data of increased ventricular iNOS expression in terminally ill patients with end-stage CHF. However, the changes in ventricular NOS activities and protein expression during the development of heart failure have not been reported. This study investigated the age-dependent alterations in ventricular NOS activities and protein expression in a genetic model of spontaneously hypertensive and heart failure-prone (SHHF) rats, in comparison to spontaneously hypertensive rats and the normotensive Sprague Dawley rats. The following hypotheses were tested:

I. Left ventricular Ca^{2+}-independent NOS activity and iNOS expression are enhanced during the development of CHF in SHHF rats, but not in SHR or SD rats.

II. Upregulation of ventricular iNOS is accompanied by an increased plasma NO_x^- level.

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CHAPTER II

ENHANCED SYSTEMIC AND PULMONARY ENDOTHELIAL NITRIC OXIDE GENERATION IN SWIM-TRAINED RATS

A version of this chapter has been submitted for publication. Rabelais Tatchum-Talom, Fadi H. Khadour. Richard Schulz and John R. McNeill.
Data in Figs. 2.1 and 2.2 are by RT-T and in Fig. 2.5 by Ken Strynadka.

INTRODUCTION

The beneficial effects of physical exercise training on cardiovascular function have been documented, although the mechanism(s) is not well understood. There is growing evidence both from animal (5, 9, 10, 13, 40) and human studies (41) suggesting that physical exercise training enhances vascular endothelial dilator function. Recently, attention has focused on the possible role of endothelium-derived nitric oxide (NO) as the main mediator of the physiological responses to exercise training. NO is synthesized by an oxidoreductase called the nitric oxide synthase (NOS). To date three isoforms of NOS have been identified. Calcium/calmodulin-dependent isoforms include neuronal NOS (nNOS, NOS I) expressed in specific neurons and in skeletal muscle (20, 33), and endothelial NOS (eNOS, NOS III) found in endothelial cells (1, 29, 35) and cardiac myocytes (3, 34). In contrast, inducible NOS (iNOS, NOS II) is a Ca^{2+}-independent enzyme and can be induced in a wide variety of cells following exposure to pro-inflammatory cytokines or bacterial endotoxin (26).

Several reports have documented diminished aortic vascular reactivity to various vasoconstrictors in experimental animals exercised by running (5, 6) or swimming (13). Studies have also shown the enhancement of endothelium-dependent, acetylcholine-mediated dilatation of the abdominal aorta in exercise trained rats (9, 10). However, most of the studies describing exercise training and vascular function in rats were performed *in vitro* on aortic rings, and little information is available regarding the effect of exercise training on endothelial vasodilator function as a whole in the intact animal. Therefore, the primary objective of the current investigation was to evaluate the effect of swim exercise training on systemic vascular reactivity in anesthetized rats *in vivo* by measuring the alterations in mean arterial blood pressure (MABP) in response to the administration of an endothelium-independent vasoconstrictor (norepinephrine) or vasodilator (S-nitroso-N-acetyl-penicillamine), and to an endothelium-dependent vasoconstrictor (N^G-nitro-L-arginine methylester) (31) or one of two endothelium-dependent vasodilators (acetylcholine, bradykinin).

A previous study by Sessa *et al.* reported the enhanced expression of eNOS mRNA in aortic endothelial cells of dogs by short-term exercise (running for 1 h, twice daily for 10 days) (36). In addition, Delp *et al.* found increased eNOS protein expression in the abdominal aortas of rats exercised by running for 4 weeks (9). However, there are no data of the combined measurements of NOS activity and expression in the aortas of trained animals. Moreover, exercise-induced changes in NOS activity and expression in the cardiopulmonary organs, which play a key role in the physiological adaptations to exercise training, have not yet been studied. Therefore, another objective of this study was to examine the changes in NOS activities and protein expression in the heart, lung and aorta caused by swim exercise training.

Our hypothesis is that physiological adaptations to swim exercise training are associated with enhanced systemic endothelial vasodilator function and NO production and this is accompanied by organ-specific upregulation of eNOS.

MATERIAL AND METHODS

All experiments and protocols were performed in accordance with the regulations of the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Saskatchewan.

Animals and swim exercise training

Studies were carried out in outbred male Sprague Dawley rats (Charles River, St. Constant, QC) of 8-10 weeks of age. Animals were kept under standard conditions in the animal house for at least 2 weeks before the training program was started. Half of the animals were randomly selected and underwent a swimming program of 60 minutes/day, 5 days/week for 3-4 weeks in a tank containing tap water approximately 50 cm deep and maintained at 20-22°C (swim-trained, n = 40). The other half were placed in a 10 cm water tank which did not require the rats to swim under the same schedule (sedentary, n = 38). On the first day of the training program, the swimming time was 10 minutes in duration, and this was then increased by 10 minutes each subsequent day until the maximum swim duration of 60 minutes was reached. On the last week, rats swam for 60 minutes twice a day, 5 days/week. Body weight and water and food intake were measured weekly before the start of swimming program and then during the entire training period.

Plasma NO_x^- levels

For determination of pre-training plasma nitrate and nitrite (NO_x^-) levels, blood (0.5 ml) was collected from the femoral artery under light ether anesthesia one week before the swimming program started. A post-training blood sample was collected one day after the last exercise episode. Blood was immediately placed in a 1.5-ml microcentrifuge tube containing 20 µl heparin sodium (10 IU/ml). Plasma was separated from blood by centrifugation at 2,000 *g* for 4 min at 4°C and stored at -20°C until analysis. After thawing, plasma was diluted 1:1 with deionized water. Then 400 µl of the diluted plasma was deproteinized by centrifugal ultrafiltration (Millipore Ultrafree-MC microcentrifuge tubes UFC3, Bedford, MA). Ultrafiltrates were analyzed for total nitrate and nitrite content by the method of Green *et al.* (12). The limit of detection was 0.1 µM for both nitrite and nitrate.

In vivo assessment of systemic vascular reactivity

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The trachea was intubated to facilitate spontaneous respiration. Body temperature was maintained at 36-37°C using a heating lamp table controlled by a rectal thermistor probe. A polyethylene cannula (PE 50, Clay Adams, Parsippany, NY) filled with heparinized saline (10 IU/ml) was inserted into the left carotid artery for the measurement of arterial blood pressure by a pressure transducer connected to a Grass polygraph (model RPS, 7C8). A second PE 50 cannula was also inserted into the left femoral vein for the i.v. administration of drugs and supplemental anaesthetic. After completing the surgery, rats were allowed to stabilize for 1 h before measurements of resting heart rate and baseline MABP were taken.

To determine the effect of swim exercise training on systemic vascular reactivity *in vivo*, the first group of rats was used to investigate the hemodynamic response (changes in MABP) to norepinephrine (NE, 0.01-10 µg/kg). Cumulative dose-response curves to i.v. bolus injections of NE were constructed at dosing intervals of 10 minutes in anesthetized rats. A second group of rats was used to examine the effects of an inhibitor of NO synthases, N^{ω}-nitro-L-arginine methylester (L-NAME, 0.01-30 mg/kg). Cumulative dose-response curves for changes in MABP to i.v. boluses of L-NAME were constructed at dosing intervals of 10-15 min, the time required to obtain a stable maximum response. A third group was studied to evaluate the effects of acetylcholine (ACh, 0.001-3 µg/kg), bradykinin (BK, 1 and 3 µg/kg) and the NO donor S-nitroso-N-acetyl-penicillamine (SNAP, 10 and 30 µg/kg). Changes in MABP were measured before, during and after each infusion. In this group, a 5-min recovery period was allowed between each dose and a 15-min recovery period between each drug.

Determination of nitric oxide synthase activities

Two additional groups of sedentary and swim-trained rats were sacrificed under ether anesthesia one day after the last swimming training. A post-training blood sample (0.5 ml) was collected by puncture of the lower abdominal aorta for determination of plasma NO_x^- levels. Then both right and left atria and ventricles, thoracic aorta and a lobe from the right lung were rapidly dissected. Excess fat and connective tissues were trimmed from the aorta. All tissues were then quickly frozen in liquid nitrogen and stored at -80°C until analysis. After measurements of atrial and ventricular wet weights, the frozen tissues were used for the assay of NOS activities as previously described (17, 18). In brief, tissues were pulverized with mortar and pestle under liquid nitrogen, homogenized and subsequently centrifuged (1000 *g*, 5 min, 4°C). The resultant supernatant was utilized for measurement of NOS activities by the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline. NOS activity was determined as the rate of citrulline production which was normalized to the protein concentration of the homogenates, as determined by the bicinchoninic acid assay using bovine serum albumin as a standard and was expressed as pmol/min/mg protein. The limit of detection was 0.1 pmol/min/mg protein.

Western blotting and densitometric analysis

Western blot analysis of tissue homogenates prepared as above was performed as previously described (18) with a few modifications. Samples (15, 12 or 7.5 μg protein per lane for lung, aorta and atrial tissues, respectively) were size-fractionated by polyacrylamide (9%) gel electrophoresis and transferred to nitrocellulose membranes by wet electroblotting. The membranes were blocked at room temperature in 10% skimmed dried milk in phosphate-buffered saline (PBS) for 3-5 h and then incubated for 1 h with murine monoclonal antibodies (Transduction Laboratories, Lexington, KY) against either human eNOS (1:4000) or human nNOS (1:2000) and thereafter with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (1:4000, Transduction Laboratories) for 1 h. The immunoreactive proteins were detected by using an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, MA). Autoradiographs were obtained by exposure to X-ray film (Kodak, X-OMAT) for 30-35s and the density of the bands was quantified by scanning densitometry using a ScanJet 6100C scanner (Hewlett Packard, Boise, ID) and SigmaGel measurement software (Jandel Corporation, San

Rafael, CA) and expressed as a percentage relative to the positive external control. As external controls for eNOS and nNOS in each blot, we used a single ventricular homogenate sample from a sedentary 8-week-old rat or rat pituitary lysate (Transduction Laboratories) as supplied by the manufacturer, respectively. Under these conditions, signal intensities were proportional to the amount of protein loaded per lane and were within the linear range of the film sensitivity.

Materials

All compounds were purchased from Sigma except L-[U-^{14}C]-arginine monohydrochloride (Amersham), L-NMMA acetate (Alexis, San Diego, CA) and bradykinin (American Peptide Co, Sunnyvale, CA). Noradrenaline bitartrate was dissolved in 0.1 mM ascorbic acid and bradykinin in physiological saline containing 0.1% bovine serum albumin.

Statistical analyses

Data are expressed as means ± SE. Values of *n* refer to the number of rats in each group. Experiments were performed according to a randomized block design. Significant differences between the groups were determined using either Student's unpaired t-test or one way analysis of variance followed by the Tukey-Kramer post hoc test. Data analysis was done using a statistical software package (SigmaStat, version 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Indices of training

Food and water intake and body weights were not statistically different in both groups of rats before swim training commenced (data not shown). The characteristics of sedentary and trained rats are summarized in Table 2.1. At the end of swim exercise training, the body weight was significantly lower in trained rats in comparison to sedentary animals ($P < 0.05$). This occurred despite the fact that both food and water intake were significantly higher in trained rats ($P < 0.05$). Atrial and ventricular weights significantly increased with swim training ($P < 0.01$). Accordingly, the ratios of atrial or ventricular weights to total body weight were markedly increased in trained rats ($P < 0.001$). Under pentobarbital anesthesia, the resting heart rate was significantly lower in trained rats than that in sedentary controls. Baseline mean arterial blood pressure was not significantly different between the groups.

Vasoreactivity of the systemic circulation

Norepinephrine dose-dependently increased MABP in both sedentary and trained rats but there were no significant differences between the two groups (Fig. 2.1, upper panel). Similarly, L-NAME produced a dose-dependent increase in MABP in both groups, however, this was significantly enhanced in trained rats at doses of 3, 10 and 30 mg/kg (Fig. 2.1, lower panel). Acetylcholine produced a short-lasting fall in MABP in a dose-dependent manner in both sedentary and trained rats. The decline in MABP was significantly greater in trained rats at doses of 0.003 and 0.01 μg/kg (Fig. 2.2, upper panel). When tested at two doses, both BK (Fig. 2.2, middle panel) and SNAP (Fig. 2.2, lower panel) reduced MABP of both sedentary and trained rats to a similar extent.

Nitric oxide synthase activity

The Ca^{2+}-dependent NOS activity in homogenates of lung, aorta, atria and ventricular tissues is depicted in Fig. 2.3. In sedentary rats, the enzyme activity was significantly different amongst the tissues in the following rank order: aorta>atria>ventricles>lung. Exercise training induced a marked increase in Ca^{2+}-dependent NOS activity in the lung (45%), aorta (45%) and atria (150%). Surprisingly, ventricular Ca^{2+}-dependent NOS activity did not change significantly with training. Ca^{2+}-independent NOS activity was not detectable in any of these tissues except for a low level in the lung. However, no significant difference was detected between sedentary and trained rats (0.20 ± 0.11 versus 0.15 ± 0.04 pmol/min/mg protein, respectively)

Western blotting and densitometric analysis

The upper panel in Fig. 2.4 shows a representative blot of immunoreactive eNOS protein in lung, aorta and atrial homogenates as resolved by 9% SDS-PAGE. By using an eNOS-specific monoclonal antibody, a single 135-kDa band was observed, corresponding to the known molecular weight of eNOS (29) in all tissues. Enhanced expression of eNOS protein was seen in lung (65%), aorta (190%) and atria (55%) of trained rats in comparison to the same tissues in sedentary animals as determined by densitometric analysis of the autoradiograms (Fig. 2.4, lower panel). No bands were seen when lung, aorta and atrial samples were incubated with anti nNOS antibody under the same conditions (data not shown).

Plasma NO_x^- levels

Before training, no significant differences in the level of plasma NO_x^- were detected between the two groups. As expected, no significant changes in NO_x^- levels were observed in rats after 3-4 weeks of sedentary living. Swim exercise training, however, also did not elicit any change in plasma NO_x^- levels (Fig. 2.5).

DISCUSSION

This study is the first to demonstrate an upregulation of Ca^{2+}-dependent NOS activities in the lung, atria and aorta of swim-trained rats. These changes in enzyme activity were accompanied by the increased expression of eNOS protein. The study also provided evidence that swim training enhances vascular endothelial dilator function as evidenced by increased responses to both an endothelium-dependent vasodilator (ACh) and endothelium-dependent vasoconstrictor (L-NAME).

The increase in atrial and ventricular weights and their ratios to total body weight, as well as the reduction in resting heart rate confirm the efficacy of the swim training protocol implemented in this study. In addition, the fact that the body weight of trained rats was significantly lower than that of sedentary controls, despite a higher food intake, suggests an increased metabolic rate and energy expenditure in swim-trained animals. These changes are in accordance with previously published data in rats exercised by running (19, 37) and are representative of the physiological adaptations of the body to regular physical exercise.

The endothelium-dependent vasodilator ACh caused a greater reduction in MABP of swim-trained rats compared with sedentary animals. In addition, enhanced pressor response was observed in trained versus sedentary rats to the endothelium-dependent vasoconstrictor, L-NAME. As NO is an important regulator of vascular tone and blood pressure (26, 31), these findings suggest that swim exercise training increases endothelial NO production in the systemic vasculature. The data are in accordance with the findings of other investigators who showed, using isolated segments of rat aorta, enhanced endothelium-dependent vasodilatation (5, 9, 10) or diminished vasoconstrictive responses to NE in trained animals via endothelium-derived NO (5, 6). These findings support the notion that the enhancement of vasodilator tone mediated by the vascular endothelium is an important mechanism for cardiovascular adaptation to regular exercise training (8).

Unlike ACh, BK, another endothelium-dependent vasodilator, reduced the MABP to the same extent in both sedentary and trained rats. BK stimulates the release from endothelial cells of a number of vasodilator factors including prostaglandins (38), NO (7) and endothelium-derived hyperpolarising factor (11). Three possibilities as to why no enhanced response to BK were seen in swim-trained rats include: 1) coupling of the BK receptor to NO production is not enhanced 2) alterations in vasoconstrictor responses to BK via BK_1 receptors on smooth muscle cells may have masked a possible enhanced NO release (mediated by BK_2 receptors) from endothelial cells and 3) the dose of BK was too high to observe differences in NO release.

Among the endothelium-independent agonists, the vasoconstrictor NE and the vasodilator/NO donor SNAP produced similar changes in MABP in both sedentary and swim-trained rats, suggesting the absence of changes in vascular smooth muscle reactivity in response to swim training.

Exercise training produced an increase in Ca^{2+}-dependent NOS activity in the lung, aorta, and atria but not the ventricles. This activity does not differentiate that from two possible sources, eNOS and nNOS. To identify which isoform was responsible for the enhanced activity, we performed Western blot analysis using monoclonal antibodies directed against eNOS or nNOS. A single immunoreactive band of 135-kDa consistent with the known molecular weight of eNOS was detected in the lung, aorta and atria. These data are consistent with observations of increased eNOS mRNA (36) and protein expression (9) in aortic extracts of trained dogs and rats, respectively. As the heart and lung play a key role in the physiological adaptations to endurance training, our data highlight the contribution of NO to this. The findings also indicate that swim exercise training upregulates the capacity of tissues to enhance the biosynthesis of NO by increasing NOS activity and/or the expression of eNOS protein. This is a possible mechanism for enhanced endothelium-dependent responses to vasoactive drugs observed in this study.

In the lung and aorta, the enhanced NOS activity and expression is presumed to be a consequence of exercise-induced increase in the cardiac output and blood flow which would cause an increase in wall shear stress in the aorta and pulmonary vessels. The endothelial cells lining these vessels sense the increase in shear stress and respond by releasing more NO (4, 22, 25, 32). When this process is of a sufficient intensity and duration, eNOS protein synthesis will also be increased (27, 30, 39). This presumption is supported by the finding of a shear stress responsive element in the eNOS gene promoter from endothelial cells (24) suggesting that NOS expression in these cells is transcriptionally regulated by shear stress. However, the fact that atrial but not ventricular NOS was enhanced with training suggests that the increased level of stretching and/or dilatation in the atria compared to ventricles (2, 28), rather than increased shear stress, is the underlying mechanism for the upregulation of NOS in the atria.

There was no Ca^{2+}-independent (inducible) NOS activity in the aorta, atria and ventricles and a low level in the lungs, which was not different between sedentary and trained rats. Furthermore, the Ca^{2+}-dependent NOS activity was due to eNOS but not nNOS, as Western blot analysis did not reveal the presence of nNOS in any of these tissues. Neuronal NOS has a very limited distribution within specific nerve fibers in the heart (33) and has not been reported to contribute significantly to cardiac Ca^{2+}-dependent NOS activity. This isoform has also been localized in the airway epithelium of rat lung (33) but the functional significance of its localization at these sites is unknown. Thus the increase in NO biosynthesis in cardiopulmonary organs apparently is specific to an upregulation of eNOS, but not nNOS or iNOS. This is in marked contrast to skeletal muscle where the enhanced NOS activity as a result of swim exercise training is due to enhanced expression of nNOS (Chapter III).

Our data indicate that the post-exercise plasma NO_x^- level in trained rats was not significantly different from the pre-exercise level. It therefore, does not substantiate the findings of Jonsdottir and co-workers who reported a significant decline in plasma nitrate level of spontaneously hypertensive rats after 7 days of exercise training. The level returned to an approximate pre-training value at 14 and 21 days of exercise, and then was increased above the pre-training level after 35 days of exercise (14). Similarly, our findings are in discordance with the report in humans of a higher resting plasma nitrate level in athletic subjects than in non-athletic controls (15). This controversy may be related to either a possible strain or species variation, the type or intensity of exercise training, or the fact that plasma nitrate level is influenced by exogenous sources such as nitrate or nitrite containing food or water (16). In our study, rats were fed with commercially available rat chow and regular tap water and no dietary restrictions were practiced. Since the plasma half-life of nitrate is 3.8 h in dogs (42) and 7-8 h in humans (16), a significant amount of nitrate may have been excreted via the kidneys in the urine or through the lung as exhaled NO gas until post-training blood samples were collected approximately one day after the last exercise episode.

Several limitations of this study warrant further discussion. First, we cannot exclude the contribution of other vasoactive factors, such as the prostaglandins which are known to contribute to the regulation of vascular tone during exercise (21). Second, systemic vascular endothelial function was evaluated via measurements of MABP changes in anesthetized animals. Consequently, the alterations in MABP might have been blunted or exaggerated under the influence of the anaesthetic. Third, the cellular source of enhanced eNOS expression in the lung, aorta and atrial tissues remains unknown and would require immunohistochemical localization. However, Ca^{2+}-dependent NOS activity and/or eNOS protein have been localized in the pulmonary vascular (23), aortic (29), coronary vascular (1) and endocardial endothelia (35), as well as in cardiac myocytes (3, 34).

In conclusion, this study provides functional and biochemical data for enhanced vascular endothelial dilator function with organ-specific upregulation of eNOS activity and expression in rats as a consequence of swim exercise training. Upregulation of NO biosynthesis in this setting may be part of flow-mediated vasodilatation (aorta, lung), or may influence myocardial contractility, O_2 consumption and/or metabolism (atria). The role of NO in exercise-induced enhancement in vasodilator tone relative to other vasoactive factors remains to be established and the physiological implications from modulating NO generation during exercise training require further study.

Table 2.1 Food and water intake, body weight, morphometric and hemodynamic parameters in sedentary and swim-trained rats.

	Sedentary	**Swim-trained**
Food intake/body wt (g/kg/day)	60.3 ± 1.2	67.0 ± 1.5*
Water intake/body wt (g/kg/day)	107.7 ± 2.1	131.8 ± 2.9*
Body wt (g)	477.0 ± 9.0	451.0 ± 9.0*
Atrial wt (mg)	104.5 ± 6.3	161.4 ± 7.6*
Atrial wt/body wt (g/kg)	0.22 ± 0.01	0.35 ± 0.02*
Ventricular wt (g)	1.20 ± 0.04	1.47 ± 0.03*
Ventricular wt/body wt (g/kg)	2.44 ± 0.02	3.08 ± 0.06*
Heart rate (bpm)	341 ± 9	309 ± 8*
MABP (mmHg)	99.6 ± 4.1	104.6 ± 4.1

Values are mean ± SE; n = 8-9 rats in each group. wt, weight; MABP, mean arterial blood pressure. * $P < 0.05$ versus sedentary controls.

Fig. 2.1 Changes in mean arterial blood pressure (MABP) induced by the i.v. administration of norepinephrine (NE, upper panel), or N^{ω}-nitro-L-arginine methylester (L-NAME, lower panel) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.

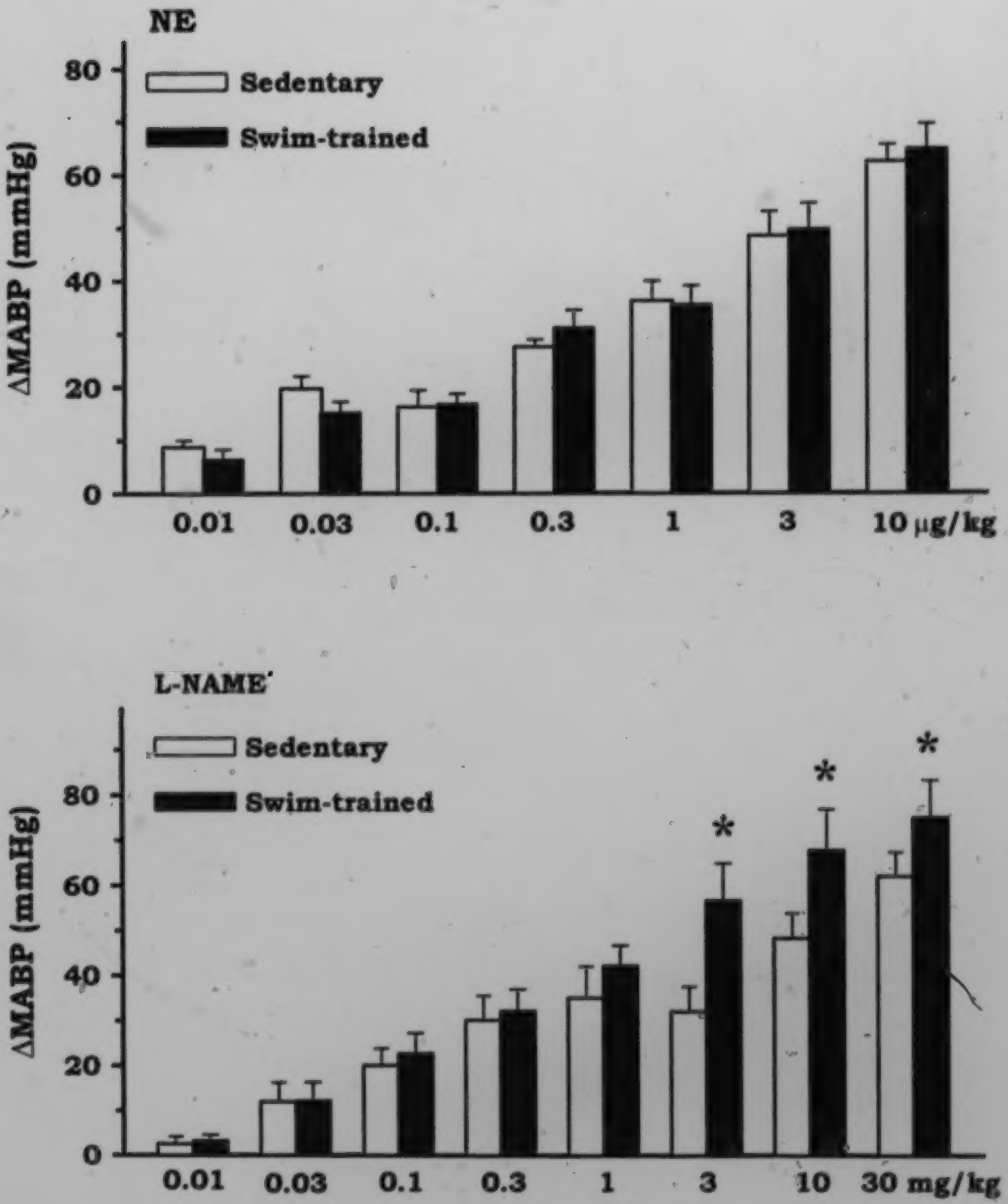


Fig. 2.2 Changes in mean arterial blood pressure (MABP) induced by the i.v. administration of acetylcholine (ACh, upper panel), bradykinin (BK, middle panel) or S-nitroso-N-acetyl-penicillamine (SNAP, lower panel) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.

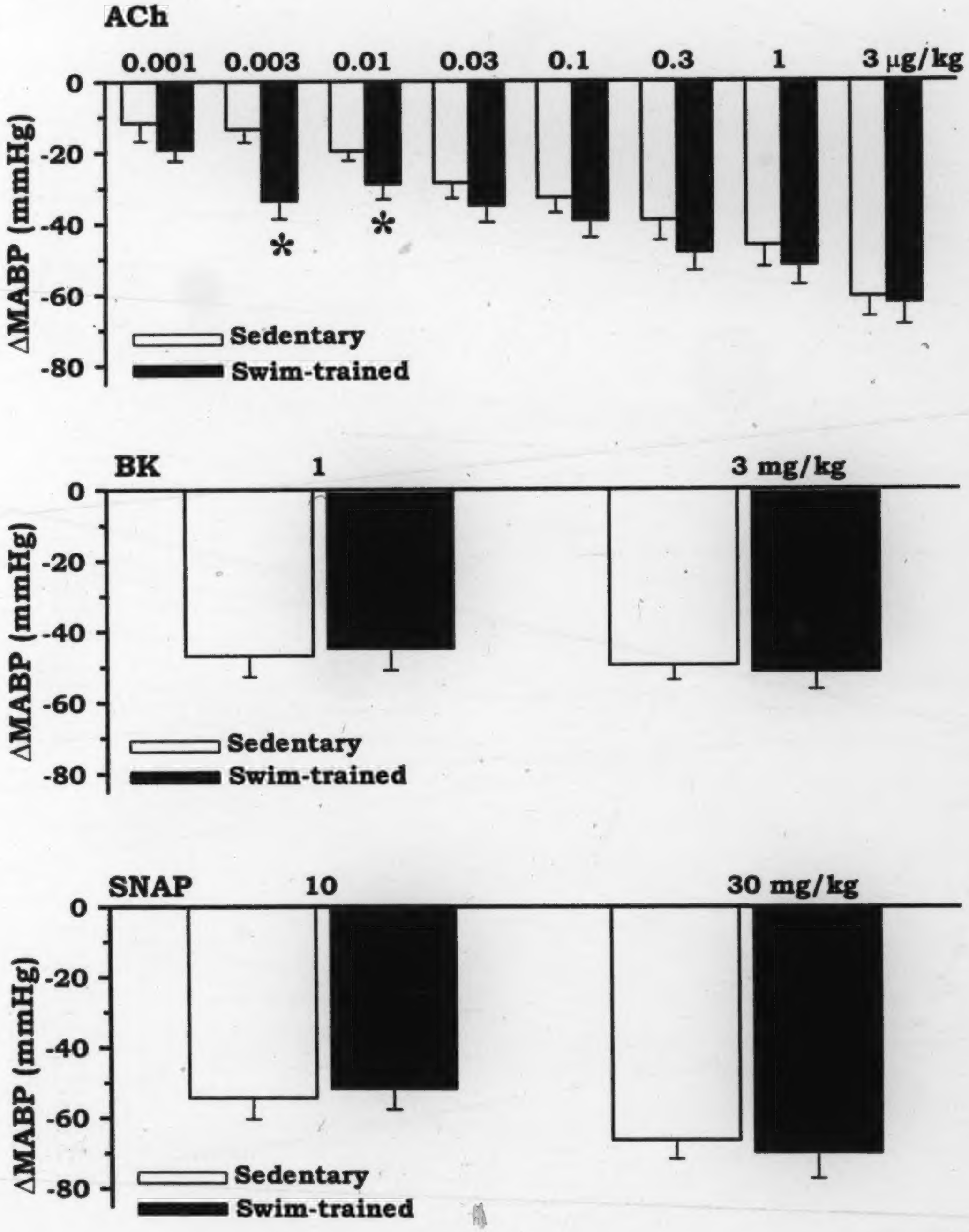


Fig. 2.3 The Ca^{2+}-dependent NOS activity in pulmonary, aortic, atrial and ventricular tissues from sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 7 per group. * $P < 0.05$ versus sedentary controls.

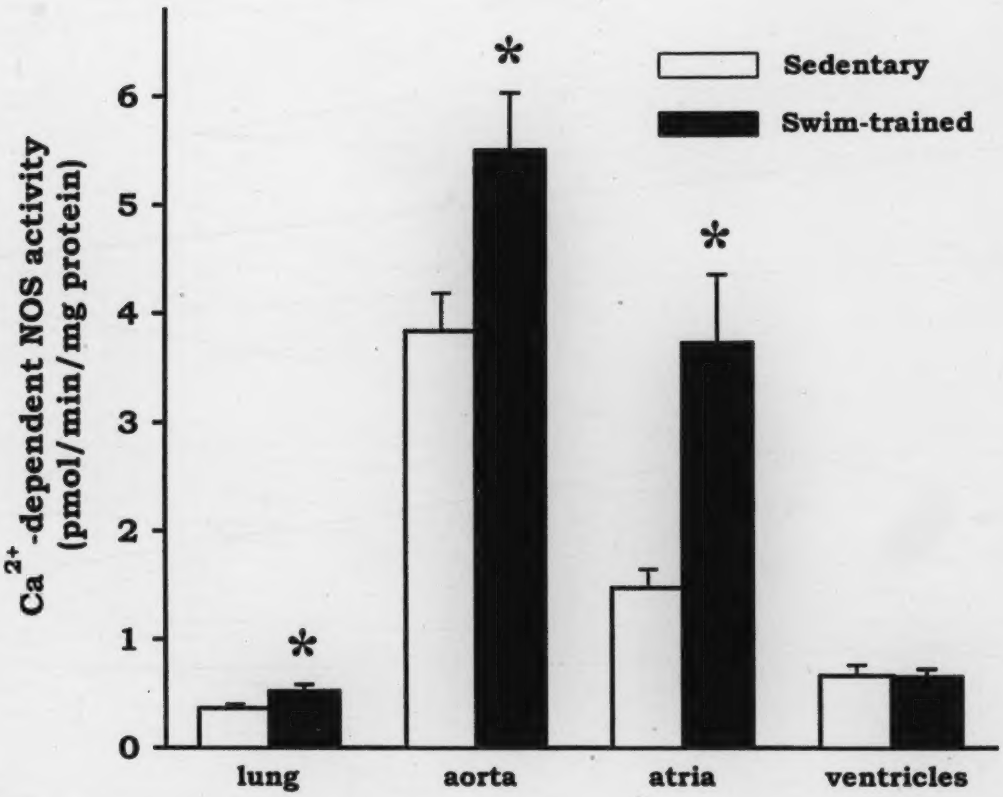


Fig. 2.4 Western blot and densitometric analysis of endothelial NOS (eNOS) in extracts of lung, aorta and atria from sedentary and swim-trained rats. **Upper panel:** Western blot of eNOS protein expression in the lung, aorta and atria; 15, 12 and 7.5 μg of protein were loaded per lane, respectively. S = sedentary rats, T = swim-trained rats. **Lower panel:** densitometric analysis of changes in eNOS protein expression in sedentary (open columns) and swim-trained rats (solid columns). Data are presented as a percent relative to the same external control run in each blot (a ventricular homogenate from a sedentary 8-week-old rat). Values are means ± SE. n = 6 per group. * $P < 0.05$ versus sedentary controls.

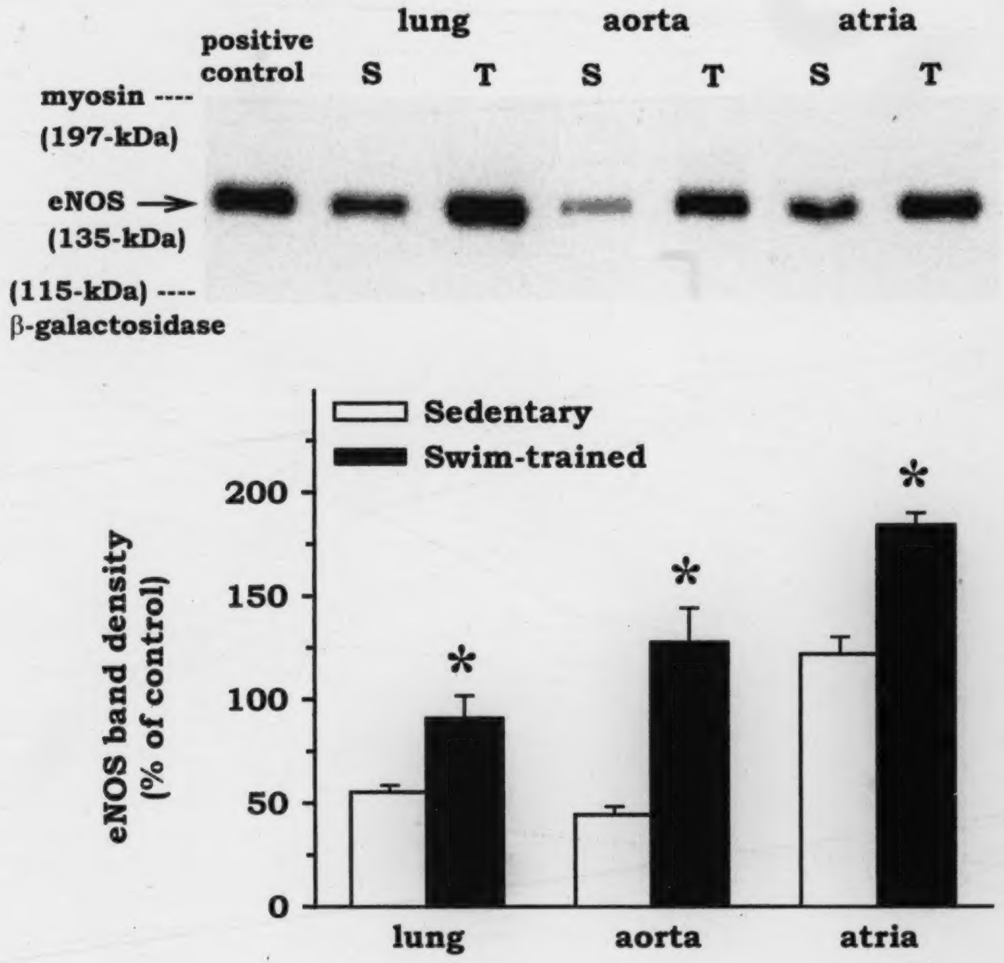
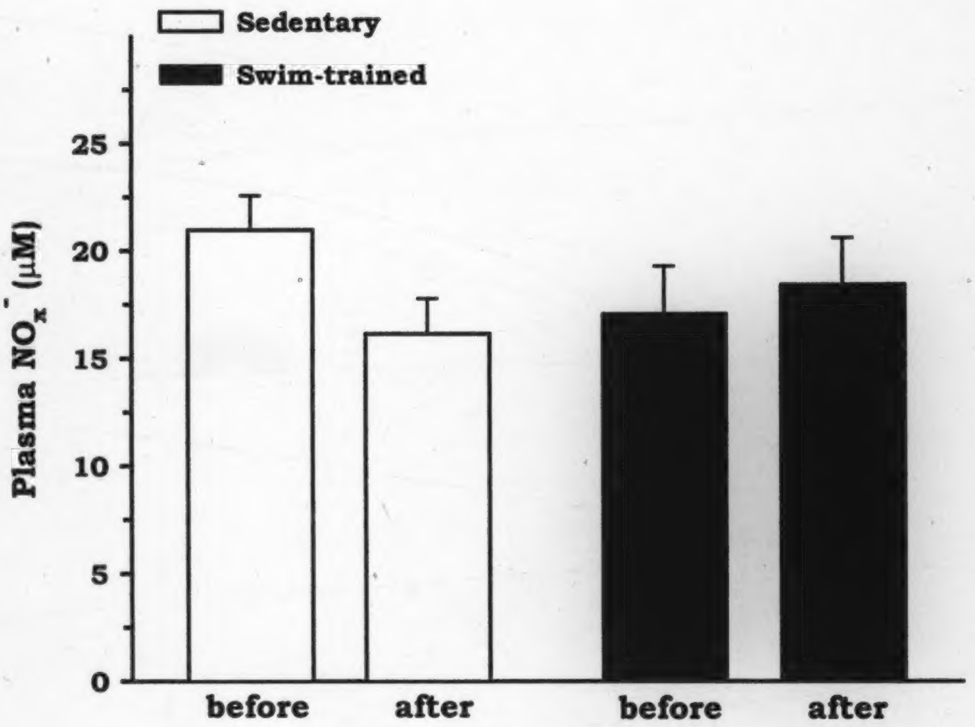


Fig. 2.5 Plasma nitrate/nitrite (NO_x^-) levels in sedentary (open columns) and swim-trained rats (solid columns) **before** and **after** 3 weeks of sedentary living or swim training. Values are means ± SE. n = 9 per group.



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CHAPTER III

UPREGULATION OF NEURONAL NITRIC OXIDE SYNTHASE IN SKELETAL MUSCLE BY SWIM TRAINING

A version of this chapter has been submitted for publication. Fadi H. Khadour, Rabelais Tatchum-Talom, John R. McNeill and Richard Schulz.
Data in Figs. 3.1-3.5 and 3.7 are by RT-T.

INTRODUCTION

The impressive increase in blood flow to the working skeletal muscle, which occurs during acute exercise, serves to augment the delivery of oxygen and nutrients and the removal of metabolic waste (24). This is accomplished by extensive vasodilatation of the arterial vasculature in skeletal muscle during exercise (24). Recent studies have focused on the possible role of endothelium-derived nitric oxide (NO) in exercise-induced dilatation of the muscle vasculature. NO is synthesized from L-arginine by an oxidoreductase called the nitric oxide synthase (NOS). To date three isoforms of NOS have been identified. Neuronal NOS (nNOS, NOS I) and endothelial NOS (eNOS, NOS III) are calcium-dependent isoforms and expressed under physiological conditions. In contrast, the activity of NOS II (iNOS), the expression of which is induced by immunological stimuli, is Ca^{2+}-independent (27). Recent reports suggest the presence of a mitochondrial isoform of NOS with characteristics intermediate between constitutive and inducible NOS (9, 18, 41).

Experimental evidence, using the radiolabelled microspheres technique, has demonstrated an enhancement of blood flow in the hindquarter skeletal muscles of exercised animals (12, 22, 28). Furthermore, Hirai and co-workers reported that NO contributes to the exercise-induced hyperemic blood flow response in rat hindquarter muscle (11). However, *in vivo* measurements of swim exercise training-induced changes in hindquarter vascular reactivity have not been performed. In addition, although Balon *et al.* reported an increased expression of both neuronal and endothelial NOS protein in the soleus muscle of rats subjected to chronic treadmill running (2), combined measurements of the alterations in NOS activity and expression, along with changes in vascular reactivity of hindquarter skeletal muscles have not yet been reported.

There are also some data suggesting that blood flow to the splanchnic and renal vascular beds is reduced during exercise (1, 11, 12, 28). This is because the increase in cardiac output is primarily directed towards the actively working skeletal muscles. However, blood flow to specific abdominal organs (kidney, liver, pancreas, stomach and intestine) is NO-mediated at least partially during exercise (11). Moreover, NO plays a role also in regulating blood flow through the mesenteric arterial bed (13, 29). To date, swim training-induced changes in NOS activity and expression in the abdominal organs, specifically the kidney and liver, are unknown and the alterations in vasoreactivity of the superior mesenteric arterial bed with training have not been studied.

We investigated swim exercise training-induced changes in hindquarter vascular reactivity in the rat by *in vivo* measurements of hindquarter blood flow and vascular conductance. The responses to an endothelium-independent vasoconstrictor (norepinephrine) or vasodilator (S-nitroso-N-acetyl-penicillamine), and to an endothelium-dependent vasoconstrictor (N^G-nitro-L-arginine methylester) (31) or one of two endothelium-dependent vasodilators (acetylcholine, bradykinin) were studied. The responses to these vasoactive drugs were also studied in a visceral vascular bed, the isolated superior mesenteric arterial bed. We also determined possible alterations in NOS activity and expression in the hindquarter skeletal muscle, kidney and liver caused by swim exercise training. We tested the hypothesis that swim exercise training alters NO-mediated vasoreactivity of the hindquarter circulation, but not that of the mesenteric arterial bed, together with enhanced activity and expression of NOS in skeletal muscle.

MATERIALS AND METHODS

All experiments and protocols were performed in accordance with the regulations of the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Saskatchewan.

Animals and swim exercise training

Studies were carried out in outbred male Sprague Dawley rats (Charles River, St. Constant, QC) of 8-10 weeks of age. Animals were kept under standard conditions in the animal house for at least 2 weeks before the training program was started. Half of the animals were randomly selected and underwent a swimming program of 60 minutes/day, 5 days/week for 3-4 weeks in a tank containing tap water approximately 50 cm deep and maintained at 20-22°C (swim-trained, n = 56). The other half were placed in a 10 cm water tank which did not require the rats to swim under the same schedule (sedentary, n = 58). On the first day of the training program, the swimming time was set at 10 minutes in duration, and this was then increased by 10 minutes each subsequent day until a maximum swim duration of 60 minutes was reached. On the last week, rats swam for 60 minutes twice a day, 5 days/week. Body weight and water and food intake were measured before the start of swimming program and then weekly during the entire period.

In vivo measurements of hindquarter blood flow and conductance

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the trachea was intubated to facilitate spontaneous respiration. Body temperature was maintained at 36-37°C using a heating lamp table controlled by a rectal thermistor probe. A polyethylene cannula (PE 50, Clay Adams, Parsippany, NY) filled with heparinized saline (10 IU/ml) was inserted into the left carotid artery for the measurement of arterial blood pressure by a pressure transducer connected to a Grass polygraph (model RPS, 7C8). A second PE 50 cannula was also inserted into the left femoral vein for the i.v. administration of drugs and supplemental anaesthetic. For hindquarter blood flow measurement, a midline laparotomy was performed, and a 1-mm ultrasonic flow probe (model 1RB; Transonic, NY) was implanted around the distal abdominal aorta. The space between the artery and flow probe was filled with inert lubricating jelly (Aquasonic gel, Parker Laboratories, NJ) which acted as an acoustic couplant. Blood flow was determined by the ultrasonic transit time shift technique as described earlier (5, 10) with the use of a small animal flowmeter (model T206; Transonic, NY). The analogue output of the flow recording was displayed on the Grass polygraph. After completion of the surgery, rats were allowed to stabilize for 1 h before measuring the following baseline cardiovascular parameters: mean arterial blood pressure, hindquarter blood flow (HQBF) and hindquarter vascular conductance (HQC, calculated as hindquarter blood flow divided by mean arterial blood pressure).

To determine the effect of swim exercise training on HQBF and HQC *in vivo*, the first group of rats was used to investigate the hemodynamic responses to norepinephrine (NE, 0.01-10 μg/kg). Cumulative dose-response curves for changes in HQBF and HQC to i.v. bolus injections of NE were constructed at dosing-intervals of 10 min in anesthetized rats. A second group of rats was utilized to examine the effects of N^{G}-nitro-L-arginine methylester (L-NAME, 0.01-30 mg/kg). Cumulative dose-response curves for HQBF and HQC to i.v.

boluses of L-NAME were constructed at dose-intervals of 10-15 min, the time required to obtain stable maximal responses. A third group of rats was used to examine the effects of acetylcholine (ACh, 0.001-3 µg/kg), bradykinin (BK, 1 and 3 µg/kg) and the NO donor, S-nitroso-N-acetyl-penicillamine (SNAP, 10 and 30 µg/kg). Changes in HQBF and HQC were measured before, during and after each infusion. A 5-min recovery period was allowed between each dose and a 15-min period between each drug.

In vitro vasoreactivity of the superior mesenteric arterial bed

On the day following the last swimming exercise, additional groups of animals were anesthetized with ether and the superior mesenteric artery was cannulated and the gut was removed as previously described (25, 40). The mesenteric arterial bed was perfused through the cannula by a pump at a constant flow rate of 5 ml/min with modified Krebs-Henseleit solution (in mM: NaCl 118, KCl 4.7, $MgCl_2.6H_2O$ 1.2, NaH_2PO_4 1.0, $CaCl_2.2H_2O$ 2.6, $NaHCO_3$ 25, glucose 11.1; 37°C; pH 7.35-7.45), oxygenated with a 95% oxygen/5% carbon dioxide gas mixture. The solution contained 1 µM indomethacin to block cyclooxygenase activity. The bed was also superfused with the same solution at a flow rate of 0.2 ml/min using a second pump connected via a T-shaped connector attached to the perfusate line. A bubble trap situated at the beginning of the superfusate system removed any air bubbles from the perfusate. Drugs were injected into the perfusate and arteriolar constriction or dilatation was determined by the changes in perfusion pressure measured with a pressure transducer (Beckman, Palo Alto, CA) placed in the perfusion circuit just before the mesenteric arterial bed. Mean perfusion pressure was recorded after electronic integration of the pulsatile pressure signal. All reported perfusion pressure values were corrected by subtracting the pressure generated by the tubing circuit of the perfusion system.

To determine the effects of swim training on the isolated mesenteric arterial bed, the first series of beds were used to study the vasoconstrictor response to norepinephrine (NE). After a 30-min stabilization period, a 0.1 ml boluses of phenylephrine (10^{-7} M) was injected over 5 seconds into the perfusate at 5 min intervals over 30 min by which time a reproducible constrictor response was obtained. A cumulative dose-response curve to NE (10^{-9} - 6×10^{-7} M, n=6-8/group) was then constructed before and after 30 minutes incubation with L-NAME (100 µM) by injecting increasing doses into the perfusion system at 5 min intervals. The second series of beds (n=8-10) was utilized to determine the vasodilator responses to ACh or SNAP. After a 30-min stabilization period, the mesenteric arterial bed was preconstricted with methoxamine (30-70 µM) for 30 min to increase the perfusion pressure to 90-100 mmHg, and then ACh was administered in the perfusate. Bolus injections of ACh (10^{-12} -6 $\times 10^{-10}$ M) were repeated with stepwise increases in the concentration at 5 min intervals. After the completion of the ACh dose-response curve, a 20-min recovery period was allowed and then SNAP (10^{-9}-10^{-6} M) was tested in the same manner.

Determination of nitric oxide synthase activities

In rats from which the mesenteric arterial bed was removed, the right kidney, liver and a sample of hindquarter skeletal muscle (left quadriceps femoris) were rapidly dissected. Excess fat and connective tissues were trimmed from the skeletal muscle and kidney. All tissues were then quickly frozen in liquid nitrogen and stored at -80°C until analysis. The skeletal muscle, hepatic and renal tissues were used for the assay of NOS activities as previously described (15, 16). In brief, tissues were pulverized with a mortar and pestle under liquid nitrogen, homogenized and subsequently centrifuged (1000 *g*, 5 min, 4°C). The resultant supernatant was utilized for measurement of NOS activities by the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline. NOS activity was measured as the rate of citrulline production which was normalized to the protein concentration of the homogenates as determined by the bicinchoninic acid assay using bovine serum albumin as a standard and was expressed as pmol/min/mg protein. The limit of detection was 0.1 pmol/min/mg protein.

Western blotting and densitometric analysis

Western blot analysis was performed as previously described (16) with a few modifications. Samples (60 μg protein per lane) were size-fractionated by polyacrylamide (9%) gel electrophoresis and transferred to nitrocellulose membranes by wet electroblotting. The membranes were blocked at room temperature in 10% skimmed dried milk in phosphate-buffered saline (PBS) for 3-5 h and then incubated for 1 h with murine monoclonal antibodies (Transduction Laboratories, Lexington, KY) against either human nNOS (1:2000) or human eNOS (1:4000) and thereafter with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (1:4000, Transduction Laboratories) for 1 h. The immunoreactive proteins were detected by using an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (Western Blot

Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, MA). Autoradiographs were obtained by exposure to X-ray film (Kodak, X-OMAT) for 30-35 s and the density of the bands was quantified by scanning densitometry using a ScanJet 6100C scanner (Hewlett Packard, Boise, ID) and SigmaGel measurement software (Jandel Corporation, San Rafael, CA) and expressed as a percentage relative to the positive external control. As external controls for nNOS and eNOS in each blot we used rat pituitary lysate (Transduction Laboratories) as supplied by the manufacturer or a single ventricular homogenate sample from a sedentary 8-week old rat, respectively. Under these conditions, signal intensities were proportional to the amount of protein loaded per lane and were within the linear range of the film sensitivity.

Materials

All compounds were purchased from Sigma except L-[U-^{14}C]-arginine monohydrochloride (Amersham), L-NMMA acetate and SNAP (Alexis, San Diego, CA), bradykinin (American Peptide Co, Sunnyvale, CA) and the salts for the Krebs- Henseleit solution which were obtained from BDH (Toronto, ON). Noradrenaline bitartrate was dissolved in 0.1 mM ascorbic acid, indomethacin in 3 mM sodium bicarbonate and bradykinin in physiological saline containing 0.1% bovine serum albumin.

Statistical analyses

Data are expressed as means ± SE. Values of *n* refer to the number of rats in each group. Experiments were performed according to a randomized block design. Significant differences between the groups were determined using either Student's unpaired t-test or one way analysis of variance followed by the Tukey-Kramer post hoc test. Data analysis was done using a statistical software package (SigmaStat, version 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Indices of training

Food and water intake and body weights were not statistically different in both groups of rats before swim training commenced (data not shown). The characteristics of sedentary and trained rats are summarized in Table 2.1 (Chapter II).

Hindquarter blood flow and conductance

Basal HQBF was significantly higher in trained rats (18.8 ± 1.7 vs. 14.4 ± 1.2 ml/min in sedentary rats, $P < 0.05$) but HQC was not different among the groups (0.19 ± 0.02 vs. 0.16 ± 0.02 ml/min in sedentary rats, $P = 0.30$). Norepinephrine induced a dose-dependent decrease in both HQBF and HQC in both sedentary and swim-trained rats but no statistically significant differences were noted between the groups (Fig. 3.1). L-NAME also caused a dose-dependent fall in HQBF and HQC in both sedentary and swim-trained rats but the reduction in both parameters was significantly greater in swim-trained rats (Fig. 3.2). Acetylcholine produced a short-lasting increase in the HQBF and HQC in both groups in a dose-dependent manner, which was enhanced in swim-trained rats compared to the sedentary controls (Fig. 3.3). When given at doses of 1 and 3 μg/kg, BK raised the HQBF and HQC in both groups, however, a more pronounced increment in these parameters was observed at 3 μg/kg in swim-trained rats (Fig. 3.4). SNAP induced an increase in HQBF and HQC in both groups at 10 and 30 μg/kg but only the higher dose resulted in an enhanced flow and conductance in swim-trained rats compared to the sedentary controls (Fig. 3.5).

Nitric oxide synthase activities

The Ca^{2+}-dependent NOS activity in homogenates of hindquarter skeletal muscle is depicted in Fig. 3.6 (upper panel). The enzyme activity increased significantly with swim training and was approximately 40% higher than that in sedentary rats. No significant differences in renal Ca^{2+}-dependent NOS activity were detected between sedentary and swim-trained rats (0.16 ± 0.05 and 0.17 ± 0.07 pmol/min/mg protein, respectively) and the activity in the liver of both groups was below detection limits. Ca^{2+}-independent NOS activity in the skeletal muscle and kidney was not detectable in either groups of rats; the activity in the liver was detectable but not significantly different between sedentary and trained rats (1.86 ± 0.79 versus 1.52 ± 0.81 pmol/min/mg protein, respectively).

Western blotting and densitometric analysis

Figure 3.6 (middle panel) shows a representative immunoblot, using the anti-human nNOS monoclonal antibody, of immunoreactive proteins in the hindquarter skeletal muscle of sedentary and trained rats as resolved by 9% SDS-PAGE. Two bands were observed, the upper one corresponding to the known molecular weight (160-kDa) of nNOS (19). At this time we do not know the identity of the lower band (estimated molecular weight 120-125 kDa). These blots demonstrate a marked enhancement of the expression of the 160-kDa nNOS in the swim-trained rats in comparison to sedentary animals. Neuronal NOS protein expression (160-kDa upper band) was semiquantitatively measured from the immunoblots by scanning densitometric analysis of the autoradiograms, as shown in the lower panel of Fig. 3.6. The density of the nNOS band was significantly increased by swim training and was 70% higher in these rats as compared to sedentary controls. No bands were seen when the resolved skeletal muscle proteins were incubated with an anti-human eNOS monoclonal antibody (data not shown).

Superior mesenteric arterial bed vasoreactivity

Constant flow perfusion of the isolated mesenteric arterial bed resulted in a steady basal perfusion pressure after a 60-minute stabilization period. Basal perfusion pressure was similar in both sedentary and swim-trained rats (21.0 ± 1.1 vs. 21.7 ± 1.3 mmHg, n=8-10 per group). NE dose-dependently increased perfusion pressure in both groups with no statistically significant difference between them (Fig. 3.7). Thirty minute incubation with L-NAME had no effect on basal perfusion pressure (23.7 ± 0.9 and 22.4 ± 1.2 mmHg in swim-trained and sedentary rats, respectively, n=8-10 per group) but it significantly potentiated the vasoconstrictor responses to NE causing a leftward shift in the dose-response curve to NE (Fig. 3.7, upper panel). However, no differences between sedentary and swim-trained rats were observed. Methoxamine raised perfusion pressure to a similar extent in both sedentary and swim-trained groups (100.0 ± 14.7 and 108.1 ± 7.0 mmHg, respectively, n=8-10 per group). ACh induced a dose-dependent fall in perfusion pressure of the methoxamine-preconstricted mesenteric arterial bed, the response was essentially similar in both groups except for the response to 6 x 10^{-11} M ACh (Fig. 3.7, middle panel) which was significantly enhanced in swim-trained rats. SNAP induced a dose-dependent fall in perfusion pressure of the methoxamine pre-constricted mesenteric arterial bed, however, no significant differences between the two groups of rats were observed (Fig. 3.7, lower panel).

DISCUSSION

This study is the first to demonstrate an upregulation of Ca^{2+}-dependent NOS activity in the hindquarter skeletal muscle of rats trained by swimming. The increased enzyme activity was associated with enhanced expression of neuronal, but not endothelial, NOS. This occurred in conjunction with augmented vascular responses in the lower limbs to both endothelium-dependent and -independent vasodilators as well as to the NOS inhibitor L-NAME suggesting an enhanced sensitivity of the vascular endothelium and/or smooth muscle as a result of swim training.

Baseline hindquarters blood flow was significantly higher in trained rats as compared to sedentary animals, probably reflecting a change in vascular tone or an altered metabolic state in the resting lower limb muscles. However, HQC in swim-trained rats was not significantly different from control rats because mean arterial blood pressure was somewhat elevated in this group, although this did not reach statistical significance.

As norepinephrine produced similar changes in HQBF and HQC in sedentary and swim-trained rats, this suggests an unaltered hindquarter vascular smooth muscle reactivity to this direct acting vasoconstrictor. In marked contrast, inhibition of NOS activity with L-NAME reduced HQBF and HQC in swim-trained rats to a higher degree than that in sedentary controls. Moreover, ACh and BK, two endothelium-dependent vasodilator agonists, induced significantly greater reductions in both parameters in swim-trained rats. These findings suggest increased sensitivity of the vascular endothelium to agonist-stimulated NO production in the lower limb as a result of swim training. This supports the hypothesis that one of the cardiovascular adaptations to chronic exercise involves the enhancement of vascular endothelium- and NO-dependent dilatation (6). A previous study by Sessa *et al.* reported the enhancement of eNOS mRNA expression in aortic endothelial cells of dogs subjected to short-term daily exercise (37). Most probably this resulted from increased blood flow to the lower limbs during exercise, with increased vascular wall shear stress and endothelial NO production (3, 20, 26, 36). We, however, cannot exclude whether changes in other endothelium-derived vasodilators such as prostaglandins or endothelium-derived hyperpolarizing factor also occurred as a result of exercise.

The enhanced vasodilator responses to the higher dose of the NO donor SNAP, which induces smooth muscle relaxation by direct activation of guanylyl cyclase, indicates that smooth muscle sensitivity to NO in the lower limb vessels may also be increased with swim training. In the literature there is evidence to suggest that intrinsic vasomotor reactivity to endothelium-independent dilators is not altered by exercise (4, 7, 17, 39). These data, however, were obtained from *in vitro* experiments on isolated segments from thoracic or abdominal aorta (4, 7, 17) or skeletal muscle arterioles (39). At the present time we do not have an explanation for this discordance but intriguing possibilities include that the metabolism of the nitrosothiol SNAP to NO may be enhanced *in vivo* in the swim-trained rat, or that pro-oxidant pathways to metabolize NO (i.e. superoxide) may be down-regulated as a result of swimming.

Swim training produced an increase in Ca^{2+}-dependent NOS activity in the hindquarter skeletal muscle, but not in the kidney, of trained rats. Because this activity measures in principle that both from eNOS and nNOS, we performed Western blot analysis using monoclonal antibodies directed against either of these to identify which NOS is responsible for the enhanced Ca^{2+}-dependent activity. Unlike previous reports that noted both eNOS and nNOS protein expression in skeletal muscles (2, 14, 18, 19, 42), no bands were observed when skeletal muscle homogenates from sedentary rats were incubated with the anti eNOS antibody but two bands were seen when an anti nNOS antibody was used. Our finding of swim training-induced enhancement of nNOS (160-kDa) protein expression in the rat's quadriceps femoris muscle confirms previous data reported by Balon *et al.* in 1997 in which chronic treadmill running of rats was found to increase neuronal NOS protein expression in the soleus muscle (2). The fact that eNOS protein was undetectable in this muscle in our hands may be due to the low level of eNOS protein in the homogenate as a result of low vascularity of the muscle sample, since eNOS has a limited distribution in skeletal muscle sarcolemma but is widely distributed in the vascular endothelium (18, 42). It is worth mentioning that the concentration of the primary antibody used in this study for the detection of eNOS by western blot analysis is much lower than that used for the localization of eNOS by immunohistochemistry (18, 42). At the present time, the identity of the lower molecular weight band (120-125-kDa) seen with the anti nNOS antibody in skeletal muscle samples from both sedentary and swim-trained rats is unknown. It may represent one of nNOS splice variants which has been identified in skeletal muscle (38) or a mitochondrial NOS that may have been labeled with the anti nNOS antibody. Notably, the expression of this band was also increased, along with the 160-kDa nNOS band, in swim-trained rats.

Neuronal NOS is highly expressed in adult human skeletal muscle (30), and is particularly concentrated in the sarcolemma of the muscle fibers (19), whereas eNOS co-localizes with mitochondria in the muscle fibers (18). Endothelium-derived NO regulates blood pressure and flow (8, 27, 31), but little is known about the role of NO in skeletal muscle. Accordingly, there are several possible explanations for why nNOS is upregulated in skeletal muscle as a result of swim training. First, muscle metabolism appears to be sensitive to NO at several sites, including glucose uptake, glycolysis and creatine kinase activity (32). Therefore, upregulation of nNOS can be part of an exercise-induced improvement in muscle glucose metabolism since a NO donor stimulated skeletal muscle glucose transport in the isolated and actively contracting rat muscle preparation (2). Of relevance, specific proteins that play a key regulatory role in control of glucose flux into skeletal muscle (GLUT-4 and hexokinase II) were also upregulated by increased contractile activity (35, 43). Second, exogenous and endogenous NO reversibly inhibit oxygen consumption and cellular respiration in isolated bovine cardiac muscle (44). Increased NO production, via nNOS, in the setting of exercise can be a physiological mechanism to limit oxygen consumption in the actively contracting muscle fibers when the demands for oxygen exceeds its supply. Third, nNOS has been localized, both pre-and post-synaptically (34), at the adult rat skeletal muscle endplate (21) and NO was reported to modulate cholinergic nerve transmission in Torpedo synaptosomes (34). Upregulation of nNOS by exercise training may be a mechanism of regulating neuromuscular conduction in skeletal muscle. Finally, because nNOS activity and protein expression are localized in type II (fast twitch), but not type I (slow twitch) fibers of skeletal muscles, and NOS activity was strongly correlated to type II fiber content of skeletal muscles (19), one may speculate that NO synthase protein expression may change when the fiber composition of the muscle is altered and the proportion of type II to type I fibers increases. However, Reiser and co-workers showed that chronic *in vivo* electrical stimulation of skeletal muscles resulted in increased activity and expression of nNOS together with a decrease in the ratio of fast to slow fibers, suggesting that nNOS expression is regulated by muscle activity and does not necessarily depend on the fast and slow twitch fiber composition of the skeletal muscle (33). This is supported by the findings of Tews *et al.* who showed decreased expression of nNOS in denervated muscles and the restoration of physiological nNOS expression by reinnervation (42). Moreover, since NO can diffuse readily over several cell diameters, enhanced production of NO via nNOS may also contribute to increased skeletal muscle vasodilator tone in swim-trained animals.

As expected, superior mesenteric bed vasoreactivity, both endothelium-dependent and –independent, and renal NOS activity were not altered by swim training. A number of investigators have demonstrated a reduced blood flow to the skin, kidneys and organs served by the splanchnic circulation during exercise together with an increased flow to the hindquarter muscles (1, 11, 12, 28). These reductions in blood flow are proportional to the relative exercise intensity and are mediated by vasoconstriction of the renal and splanchnic circulations (23). Accordingly, the stimulus which likely enhanced hindquarter vascular sensitivity (increased wall shear stress) is not operative in these vascular beds and this may explain the lack of changes in mesenteric arterial bed vasoreactivity and NOS activity in the kidney.

Several limitations of this study warrant further discussion. First, we have not identified the cellular site of enhanced nNOS expression in skeletal muscle, which requires immunohistochemical localization. Second, the changes in NOS activity in the abdominal organs were only determined in the kidney and liver. Additional organs and tissues, such as intestine, spleen and mesentery have to be investigated to obtain a more comprehensive and accurate view about the changes in NO production in the viscera. Third, a full dose-response curve for BK and SNAP may be necessary to demonstrate the complete range of their possible effects on HQBF and HQC. Finally, swim training-induced alterations in other vasoactive factors in the mesenteric circulation, such as endothelium-derived hyperpolarising factor should also be investigated.

In conclusion, this study reveals that long-term swim training in rats enhances the sensitivity of the hindquarters vascular endothelium and smooth muscle together with upregulation of both nNOS activity and protein expression in hindquarters skeletal muscle. Superior mesenteric bed vasoreactivity was not changed with training, nor was there a change in renal NOS activity. The mechanism by which nNOS is upregulated by swim training and the physiological significance of enhanced NO production in the skeletal muscles of trained animals requires further study.

Fig. 3.1 Changes in hindquarters blood flow (HQBF, upper panel) and conductance (HQC, lower panel) in response to the cumulative i.v. administration of norepinephrine (NE) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group.

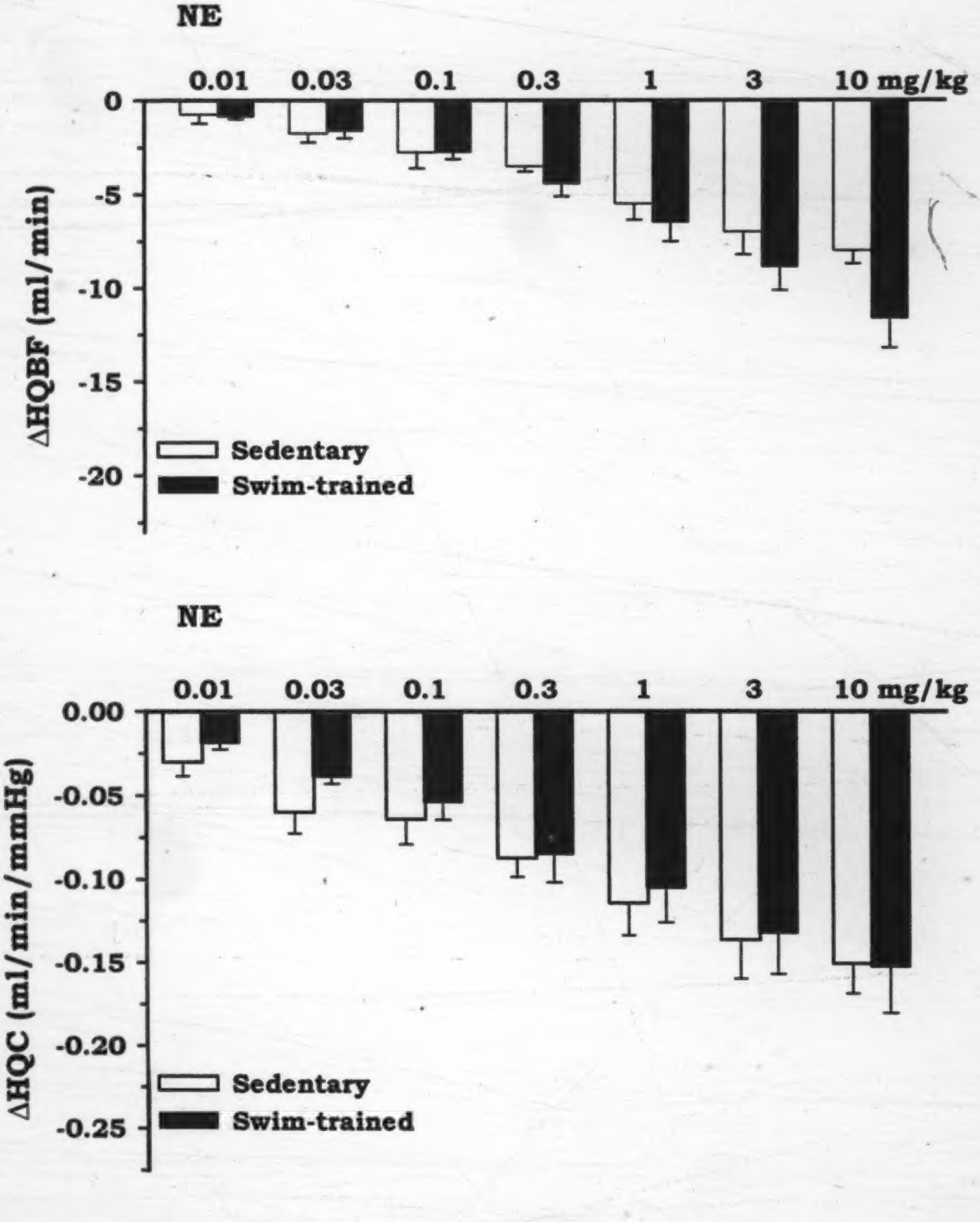


Fig. 3.2 Changes in hindquarters blood flow (HQBF, upper panel) and conductance (HQC, lower panel) in response to the cumulative i.v. administration of N^G-nitro-L-arginine methylester (L-NAME) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.

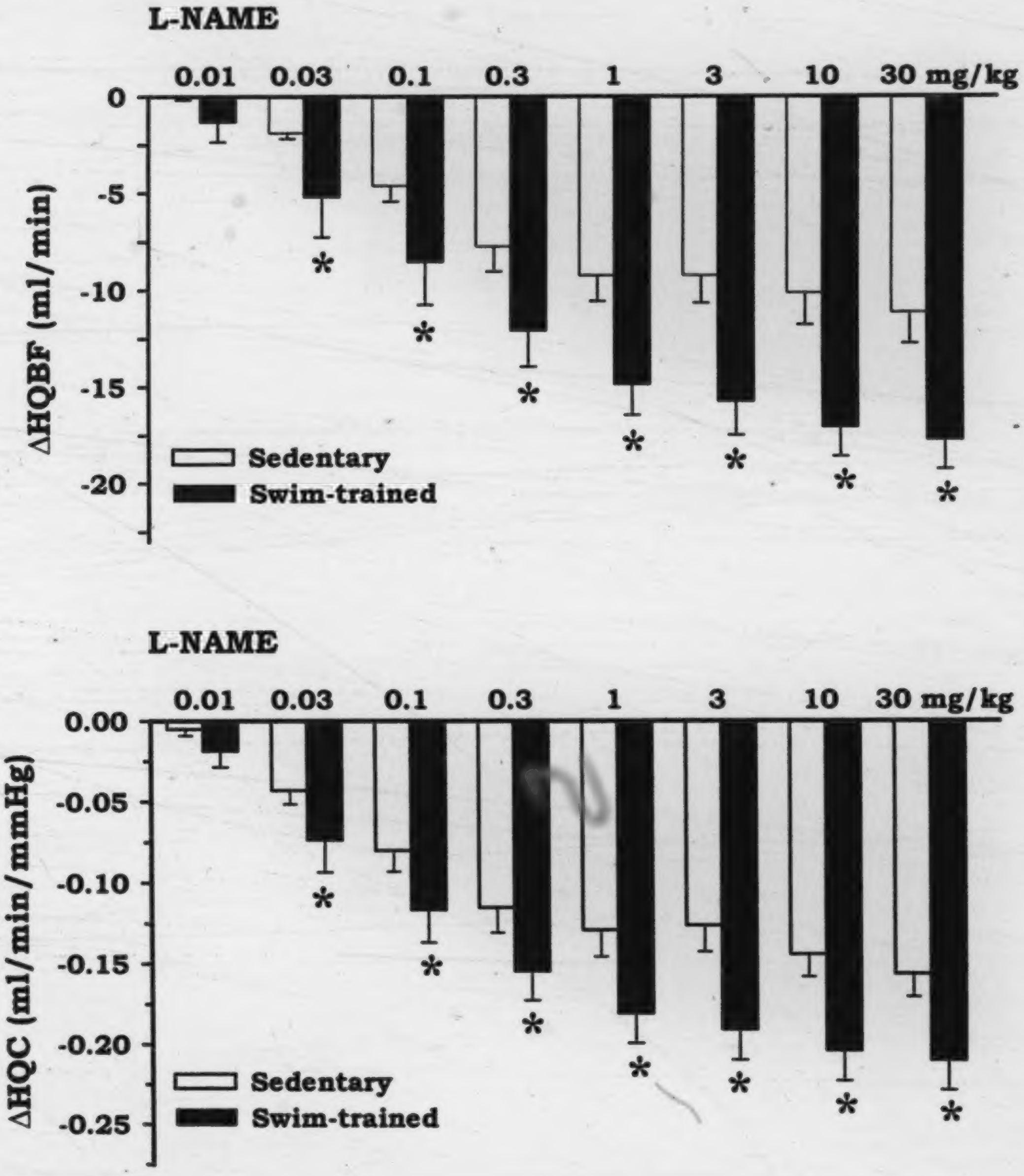
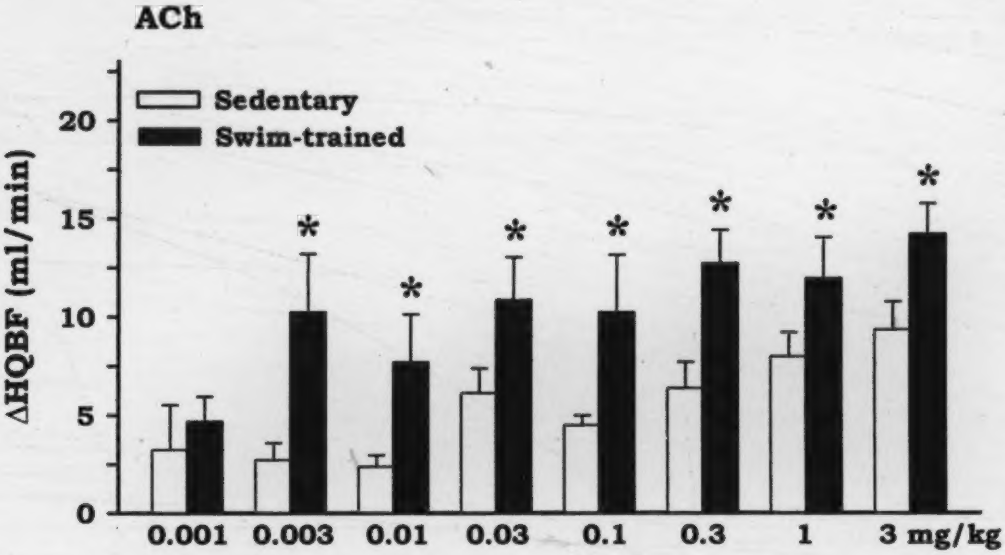


Fig. 3.3 Changes in hindquarters blood flow (HQBF, upper panel) and conductance (HQC, lower panel) in response to the cumulative i.v. administration of acetylcholine (ACh) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.



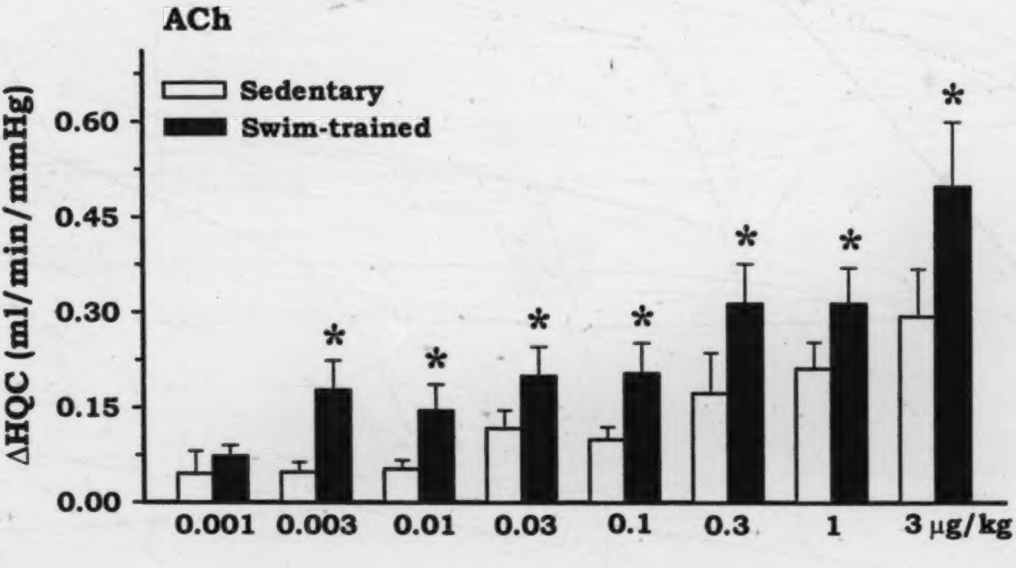


Fig. 3.4 Changes in hindquarters blood flow (HQBF, upper panel) and conductance (HQC, lower panel) in response to the cumulative i.v. administration of bradykinin (BK) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.

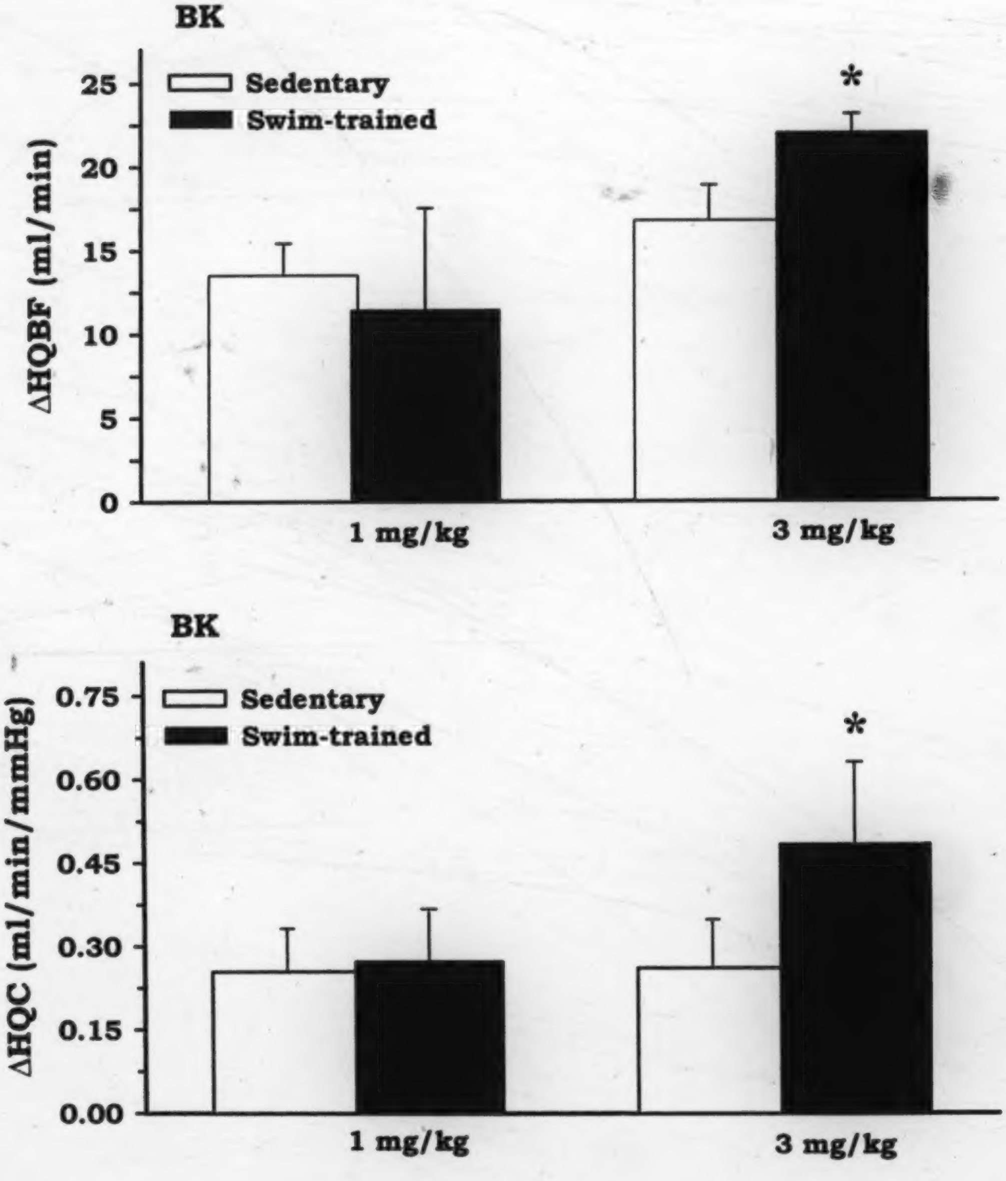


Fig. 3.5 Changes in hindquarters blood flow (HQBF, upper panel) and conductance (HQC, lower panel) in response to the cumulative i.v. administration of S-nitroso-N-acetyl-penicillamine (SNAP) in sedentary (open columns) and swim-trained rats (solid columns). Values are means ± SE. n = 8-10 per group. * $P < 0.05$ versus sedentary controls.

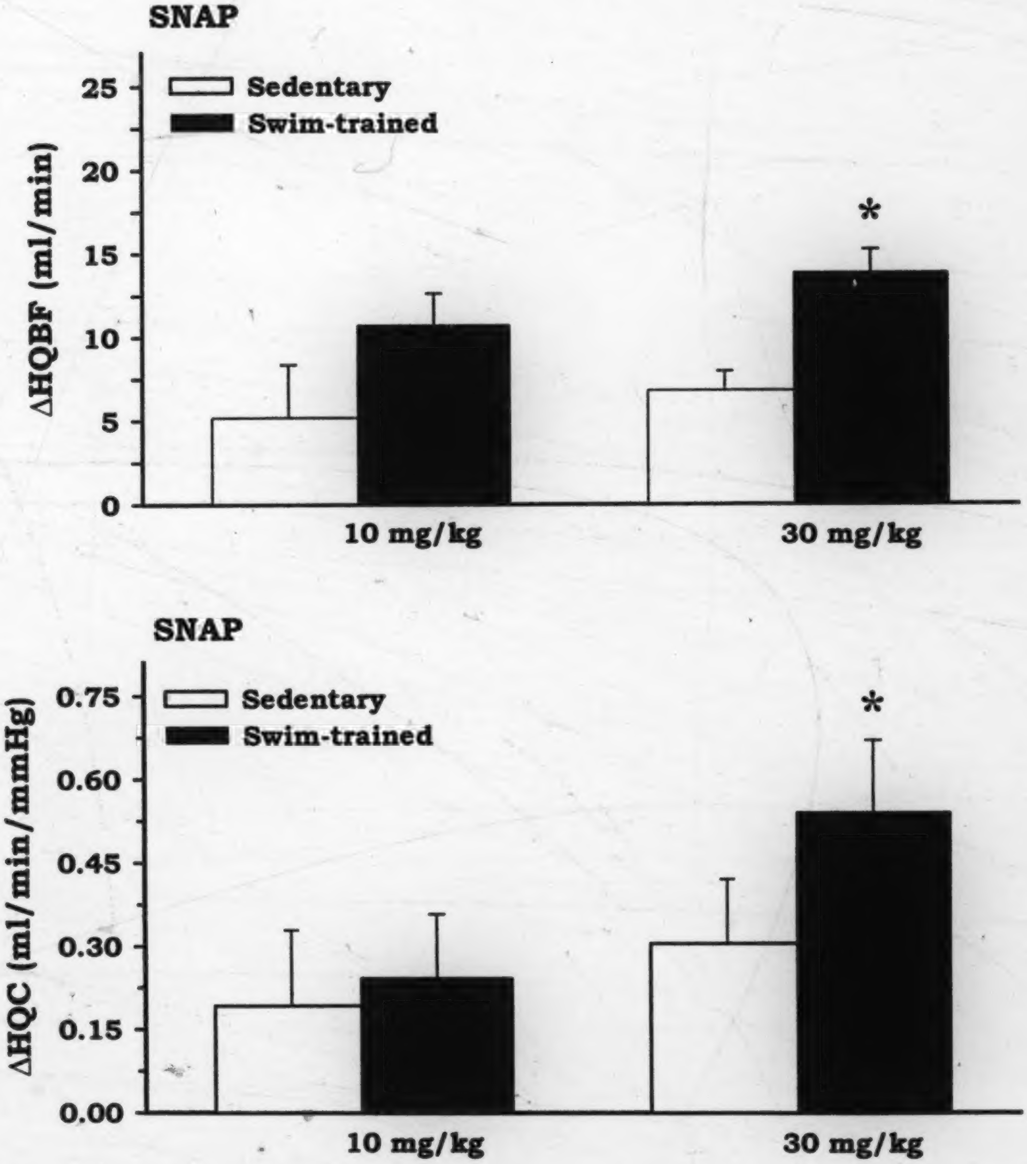


Fig. 3.6 Changes in Ca^{2+}-dependent NOS activity and neuronal NOS (nNOS) protein expression in homogenates of hindquarter skeletal muscle (left quadriceps femoris) of sedentary (open columns) and swim-trained rats (solid columns). **Upper panel:** Ca^{2+}-dependent NOS activity. **Middle panel:** Western blot of nNOS protein expression; 60 μg protein was loaded per lane. **Lower panel:** densitometric analysis of changes in nNOS protein expression. Data are presented as a percent relative to the external control for nNOS (rat pituitary lysate). Values are means ± SE. n = 5-6 per group. * $P < 0.05$ versus sedentary controls.

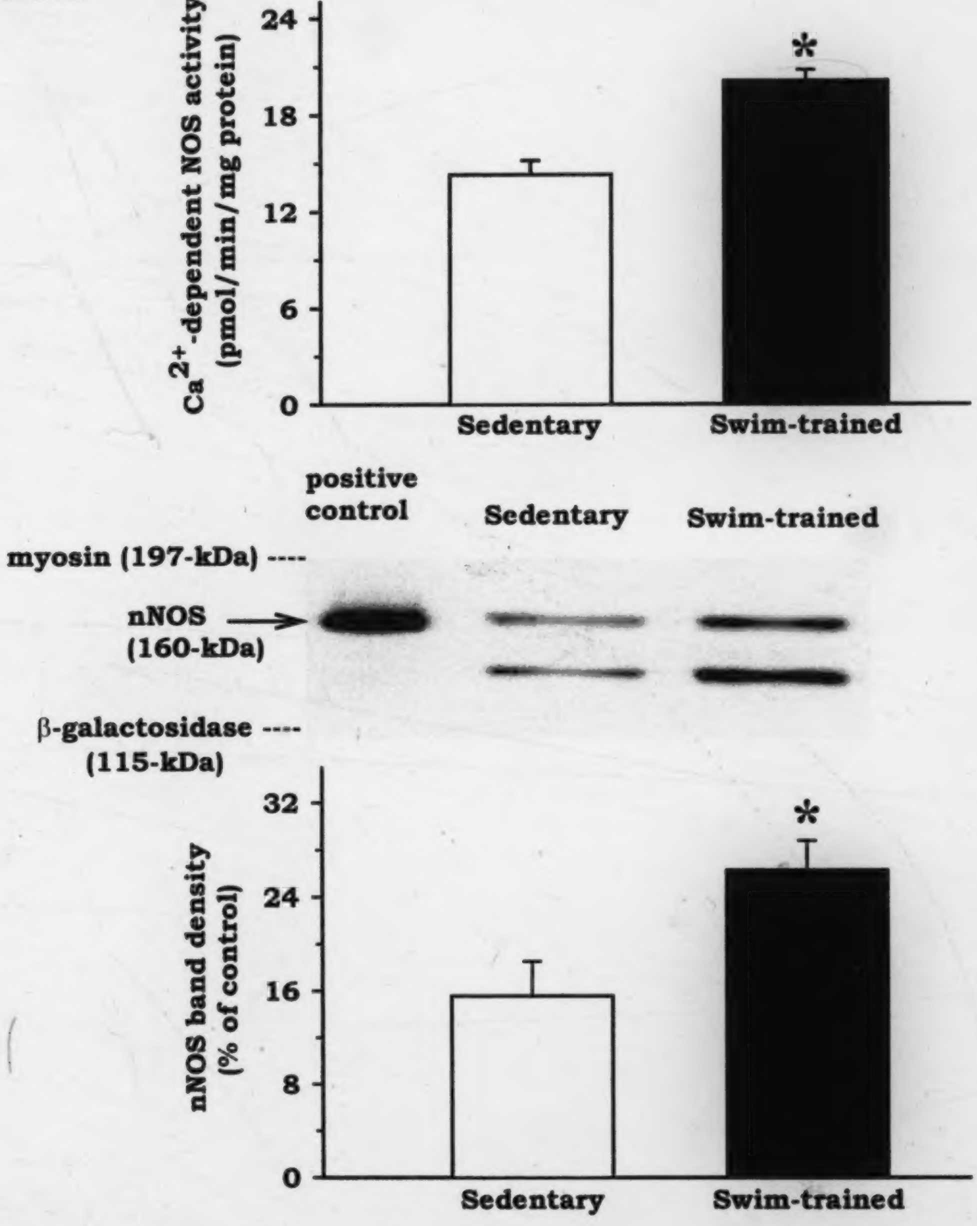
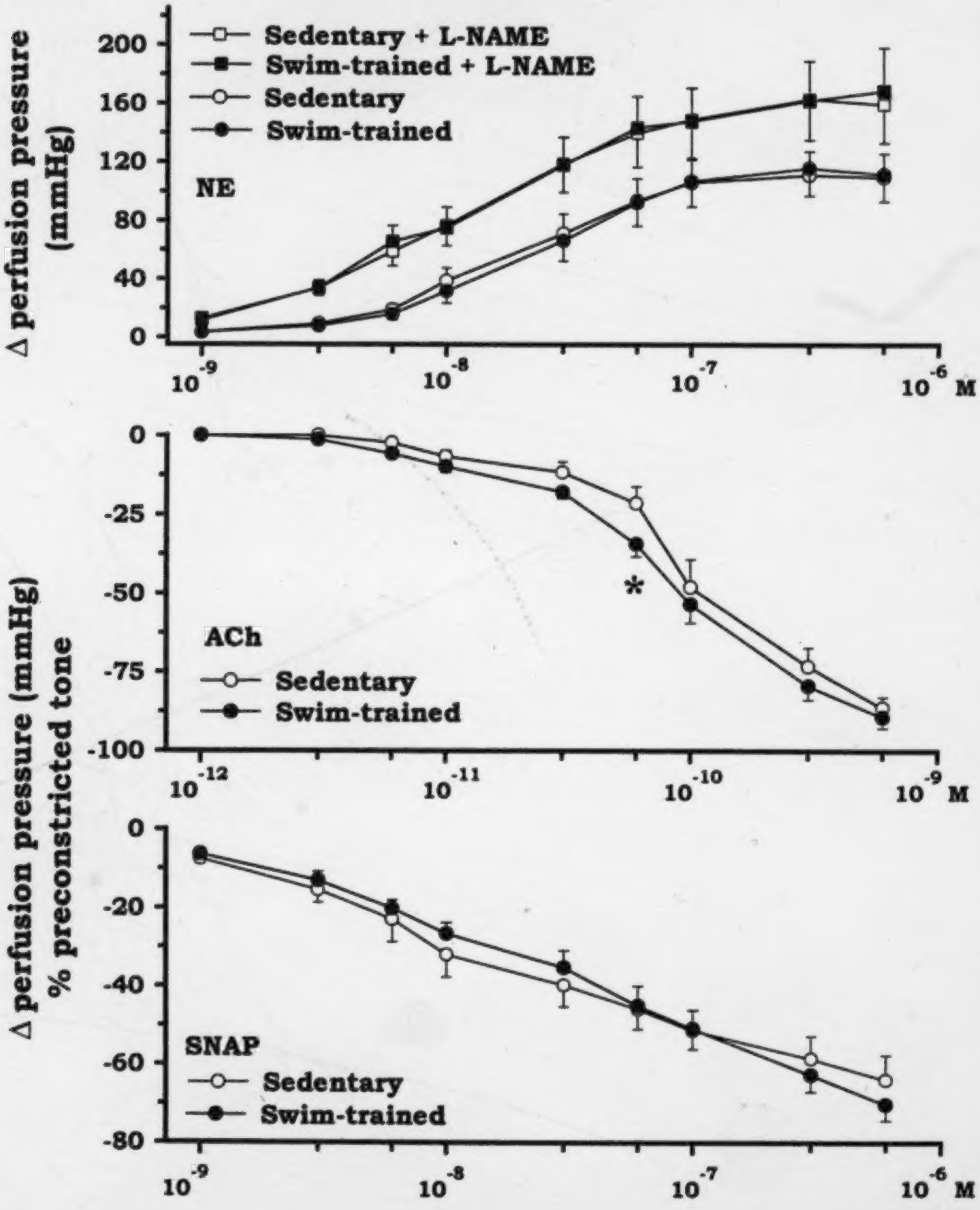


Fig. 3.7 Changes in perfusion pressure of the isolated superior mesenteric arterial bed in response to the administration of vasoactive drugs in sedentary and swim-trained rats. **Upper panel:** vasoconstrictor effect of norepinephrine (NE) administered before and after 30-min incubation with N^G-nitro-L-arginine methylester (L-NAME). **Middle panel:** vasodilator effect of acetylcholine (ACh) in the methoxamine-preconstricted bed. **Lower panel:** vasodilator effect of S-nitroso-N-acetyl-penicillamine (SNAP) in the methoxamine-preconstricted bed. Values are means ± SE. n = 6-10 per group. * $P < 0.05$ vs. sedentary controls. If not shown, error bars are within the height of the symbol.



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CHAPTER IV

ENDOTHELIAL NITRIC OXIDE SYNTHASE INCREASES IN LEFT ATRIA OF DOGS WITH PACING-INDUCED HEART FAILURE

A version of this chapter has been published. Fadi H. Khadour, Darryl W. O'Brien, Yuling Fu, Paul W. Armstrong, and Richard Schulz. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1971-H1978, 1998.
Data in Tables 4.1 & 4.2 are by DWO & YF and in Fig. 4.1A by Ken Strynadka.

INTRODUCTION

Congestive heart failure (CHF) is a complex syndrome of myocardial dysfunction and diminished cardiac output. Experimental and clinical research indicates that an alteration in cardiac nitric oxide (NO) production may play a role in the pathogenesis of CHF. Three isoforms of nitric oxide synthase (NOS) which catalyze NO biosynthesis from L-arginine have been demonstrated in the heart. There are two constitutively expressed Ca^{2+}-dependent isoforms: endothelial NOS (eNOS) located in the coronary vascular endothelium (1), endocardial endothelium (32) and cardiac myocytes (5, 31), and a neuronal isoform (nNOS) that has been localized in some cardiac neurons (38). An inducible, Ca^{2+}-independent isoform (iNOS) is expressed following exposure of the heart to bacterial endotoxin or pro-inflammatory cytokines (23, 31). The constitutive production of NO in the heart plays physiological roles in regulating coronary blood flow (1) and modulating contractile function (35) by exerting negative inotropic (5, 6, 9) and chronotropic (6, 15) actions. Upregulation of cardiac NO biosynthesis, through iNOS expression, causes a pathophysiological depression in myocardial contractile function (4, 30, 39) and may have a direct cytolytic effect on cardiac myocytes (28).

In a study of heart failure patients, Winlaw *et al.* observed that plasma levels of nitrate, a stable end product of endogenous NO metabolism, were elevated when compared to healthy individuals (42). Subsequently, the same investigators reported that the increase in plasma nitrate levels positively correlated with the severity of heart failure (43). However, measurements of cardiac iNOS in human CHF have revealed conflicting results. DeBelder *et al.* found a Ca^{2+}-independent NOS activity in right ventricular myocardial biopsy specimens of patients with dilated cardiomyopathy and myocarditis but only a Ca^{2+}-dependent NOS activity in those with ischemic and valvular heart diseases (11, 12). Expression of iNOS mRNA and protein in ventricles was reported by Haywood *et al.* in heart failure resulting from ischemic and valvular heart diseases, as well as in dilated cardiomyopathy (16). In contrast, Thoenes *et al.* detected iNOS protein in failing hearts from patients in septic shock only (40). None of these studies, however, measured the activity of iNOS (16, 40) nor examined possible changes in eNOS activity and protein expression or plasma nitrate levels (11, 12, 16, 40). Furthermore, some investigators used right ventricular tissues while others reported findings with left ventricular samples. There are no data regarding expression of NOS in the atria of normal or failing hearts, however, this would be of interest as NO has many potential effects on atrial function and structural integrity for reasons given above.

We hypothesized that ventricular NO production is enhanced, via iNOS expression, during the development of pacing-induced CHF in dogs. This model of heart failure, induced by rapid ventricular pacing at 250 beats per minute for 21 days, produces clinical, hemodynamic and neurohumoral changes similar to those found in humans (2). In normal dogs, dogs subjected to 2 days of pacing, and those to 21 days of pacing (severe CHF) our objectives were to determine: a) NO production via measurement of plasma nitrate/nitrite (NO_x^-), b) the profile of changes in Ca^{2+}-dependent and -independent NOS activities in the left atria in comparison with those in the left and right ventricles and c) the chamber-specific isoform(s) of NOS responsible for these changes.

MATERIALS AND METHODS

This study was approved and conducted in accordance with the Health Sciences Animal Welfare Committee of the University of Alberta and the Canadian Council of Animal Care. The study group consisted of twenty adult male mongrel dogs weighing 23 ± 1 kg. All dogs were conditioned to the laboratory environment at least 2 days before the beginning of the experiment. Each dog was housed in an individual cage in a room with a temperature of 20°C and a light cycle of 07:00-18:00 h. Animals were fed a fixed diet of commercially available dog food once per day in the morning and had free access to tap water. Dogs were randomly divided into 3 groups: a control group which was not paced (n = 8), dogs paced for 2 days (n = 5), and a third group which was paced for 21 days into heart failure (n = 7).

Induction of heart failure

Heart failure was induced by right ventricular pacing using a protocol that is known to produce changes in hemodynamic parameters indicative of the development of heart failure (2, 13, 29). In brief, a programmable pulse generator (Medtronic Minex-8341, Medtronics Inc., Mississauga, ON) was inserted into a subcutaneous cervical pocket and a unipolar pacemaker lead was positioned into the right ventricular apex under general anesthesia with halothane. Each dog was implanted with an externalized chronic indwelling catheter placed in the aortic arch that was used for blood sampling and pressure measurement during hemodynamic studies. Catheter patency was maintained by flushing every other day with saline followed by heparin (1.5 ml, 1000 IU/ml). Dogs were allowed to recover from surgery for 6-7 days prior to the beginning of ventricular pacing. For the induction of CHF, the pulse generator was programmed to 250 beats per minute using a Medtronic 9710A Programmer. Once pacing was initiated, daily clinical assessment was performed including observation of animal behavior, body weight, heart rate, respiratory rate, dyspnea, gingival cyanosis and ascites.

Echocardiographic and hemodynamic measurements

Hemodynamic and echocardiographic measurements were performed simultaneously at baseline during sinus rhythm just before the initiation of pacing and subsequently within 30 min after cessation of pacing. Dogs paced for 21 days were also evaluated by echocardiography at the end of the first week of pacing. Transthoracic echocardiographic studies were carried out using a Sonos 1000 series echocardiographic image system (Hewlett-Packard Medical Products Group, Andover, MA) with a 3.5 or 5 MHz transducer as previously described (13) and were used to determine left ventricular weight and ejection fraction as well as left atrial and ventricular cross-sectional areas. Hemodynamic studies were performed on sedated animals in the right decubitus position. Intravenous morphine boluses of 1-2 mg each (total dose of 4-14 mg) were given as necessary for sedation. A Swan-Ganz balloon-tipped thermodilution catheter (7F) and a high-fidelity, microtransducer-tipped pigtail Millar catheter (Millar Instruments Inc., Houston, TX) were introduced via the femoral vein (Swan-Ganz) and artery (Millar) to the pulmonary artery and left ventricle, respectively. Right atrial pressure and left ventricular end-diastolic pressure were recorded on a data acquisition system (Dataq Instruments Inc., Akron, OH). Cardiac output was determined by the thermodilution technique

(COM-2, Baxter-Edwards, Santa Ana, CA), as previously reported (13). After the final hemodynamic and echocardiographic assessments, dogs were anesthetized with pentobarbital (50 mg/kg i.v.), the chest was opened and the heart was removed. The left atrial appendages were dissected out and weighed, and tissue samples from the left ventricle (cardiac apex), right ventricular free wall and left atrium were taken. The endocardium was removed from left atrial samples by scraping the luminal surface with a scalpel blade and from ventricular tissues by removing the inner layer to an approximate depth of 3-4 mm. Myocardial tissues were then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Blood sampling and measurements of plasma NO_x^- and creatinine

Blood samples were withdrawn from the aorta just before the initiation of pacing and subsequently within 30 min after cessation of pacing. Dogs paced for 21 days were also sampled at the end of the first week. The plasma was separated from blood by centrifugation at 2,000 *g* for 5 min at 4°C and stored at -80°C until analysis. After thawing, plasma was diluted 1:1 with deionized water. Then 400 µl of the diluted plasma was deproteinized by centrifugal ultrafiltration (Millipore Ultrafree-MC microcentrifuge tubes UFC3, Bedford, MA). Ultrafiltrates were analyzed for total nitrate and nitrite (NO_x^-) content by the method of Green *et al.* (14). The limit of detection was 0.1 µM for both nitrite and nitrate. Renal function was measured by determination of plasma creatinine levels using an automated colorimetric method based on Jaffe's reaction at the University of Alberta hospital laboratory.

Preparation of atrial and ventricular homogenates for determination of NOS activities

The frozen atrial and ventricular myocardial tissues were used for the assay of NOS activities as previously described (19, 30, 31). First, tissues were pulverized in liquid nitrogen, homogenized and subsequently centrifuged (1000 *g*, 5 min, 4°C). Then, the resultant supernatant was utilized for measurement of atrial or ventricular NOS activities by the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline. Citrulline production was normalized to the protein concentration of the homogenates, as determined by bicinchoninic acid assay utilizing bovine serum albumin as a standard, and was expressed as pmol/min/mg protein. The limit of detection was 0.1 pmol/min/mg protein.

Western blotting and densitometric analysis

Western blot analysis was performed as previously described (19). Sample proteins (50 μg per lane) were size-fractionated by SDS-PAGE (7 and 9%) and transferred to nitrocellulose membranes by wet electroblotting for 170-180 min. In addition to the prestained SDS-PAGE protein standards (Bio-Rad, Hercules, CA), a positive control of recombinant bovine endothelial NOS (Cayman Chemical, Ann Arbor, MI) was utilized. The membranes were blocked at room temperature in 3% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 3 hr and then incubated with rabbit polyclonal antibody directed against bovine eNOS (Affinity Bioreagents Inc., Golden, CO; prepared in 1% BSA in TBS-T at 1:1000 dilution) for 1.5 hr and then washed with TBS-T three times (5 min each). This antibody detects canine eNOS but does not cross-react with either iNOS or nNOS. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted at 1:2000 with 1% BSA in TBS-T for 60 min, then washed three times with TBS-T and finally with phosphate-buffered saline alone (5 min each). Immunoreactive proteins were detected by using an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (ECL, Amersham, Oakville, ON). Autoradiographs were obtained by exposure to X-ray film (Kodak, X-OMAT) for one second and the density of the bands was quantified by scanning densitometry as previously reported (19) and expressed as a percentage of the positive control.

Materials

All other reagents were purchased from Sigma except L-[U-^{14}C]-arginine monohydrochloride (Amersham) and L-NMMA acetate (Alexis, San Diego, CA).

Statistical analyses

Data are presented as means ± SE of samples from *n* separate experimental animals. One way analysis of variance followed by Tukey's post hoc test was used to evaluate differences between the groups. Data analysis was done using a statistical software package (SigmaStat, version 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Congestive heart failure induced by rapid ventricular pacing in dogs

Dogs paced for 2 days showed only an increase in heart rate (sinus rhythm) and a decrease in left ventricular dP/dt and ejection fraction (Table 4.1). In contrast, dogs paced for 21 days showed an increase in left ventricular end diastolic pressure, right atrial pressure, pulmonary capillary wedge pressure and heart rate with a decrease in left ventricular systolic pressure and dP/dt, mean arterial pressure, cardiac output and ejection fraction consistent with the induction of CHF (2, 13, 29). The body weight of the dogs did not significantly change from the pre-pacing state of 21.0 ± 1.7 kg (n = 12) to 22.7 ± 1.1 kg (n = 5) after 2 days and 21.4 ± 1.6 kg (n = 7) after 21 days of pacing. As shown in Table 4.2, left atrial appendage weight doubled after 21 days of pacing in comparison to control and 2 day values. However, left ventricular mass and total heart weight remained unchanged between control and paced dogs. Accordingly, the ratio of left atrial appendage weight to heart weight increased significantly after 21 days of pacing compared to controls and to dogs paced for 2 days. A significant dilatation of the left atrium occurred as early as 2 days of pacing and increased as pacing continued until 21 days, as evidenced from the data of left atrial cross-sectional area (Table 4.2). Left ventricular cross-sectional area was significantly first elevated after 7 days and remained at this level after 21 days of pacing.

Plasma NO_x^- and creatinine levels

Figure 4.1 shows the changes in plasma NO_x^- and creatinine levels with ventricular pacing in dogs. Before pacing was started, the mean plasma NO_x^-

level was 19 ± 1 μM (n = 12). After 2 days of pacing it was unchanged from control, then it more than doubled after 7 days of pacing (Fig. 4.1A). After 21 days of pacing, plasma NO_x^- remained significantly higher than pre-pacing and 2 day values. These changes in plasma NO_x^- levels occurred in the absence of any significant alteration in plasma creatinine levels (Fig. 4.1B).

Atrial and ventricular NOS activities

The changes in Ca^{2+}-dependent NOS activity in different chambers of the heart, in control and paced dogs, are shown in Fig. 4.2. Left atrial tissues from control dogs had Ca^{2+}-dependent NOS activity of about 0.4 pmol/min/mg protein. This was significantly higher than the activity in both left and right ventricular tissues from the same animals. Moreover, the left atrial activity significantly increased in dogs paced for 21 days but not in those paced for 2 days only. In marked contrast to these findings, no significant changes in left or right ventricular Ca^{2+}-dependent NOS activities were detected in dogs paced for 2 or 21 days. Thus after 21 days of pacing the enzyme activity in the left atria was approximately 3.5 fold higher than that in either left or right ventricle. Ca^{2+}-independent NOS activity was not detectable in any of these tissues (data not shown).

Western blot and densitometric analysis

Figure 4.3 shows representative blots of immunoreactive eNOS proteins from the heart chambers of control, 2 and 21 day paced dogs as resolved by 7% SDS-PAGE. In left atrial tissues, three distinct bands can be seen in the region corresponding to the known molecular weight of eNOS. Based on studies of eNOS in rat cardiac myocytes (7), the upper and middle bands from the left atrial tissues represent the 150- and 135-kDa eNOS proteins, respectively. At this time we do not know the identity of the lowest band (estimated molecular weight 120-125-kDa). These blots demonstrate a marked enhancement of expression of the 150-kDa eNOS protein in the left atrium concomitant with the development of heart failure after 21 days of pacing. In left and right ventricular tissues, only 2 bands were seen corresponding to the 150- and 135-kDa eNOS proteins. The density of these two bands did not change significantly with pacing. Triplet atrial and doublet ventricular eNOS bands were also detected in a similar fashion using 9% SDS-PAGE in the current study (data not shown). As depicted in Fig. 4.4, densitometric analyses of all hearts subjected to Western blotting show enhanced expression of the 150-kDa eNOS band at 21 days of pacing in left atrial tissues. This was associated with a significant increase in total eNOS expression, whereas no significant changes were noted in the 135-kDa band. The ratio of 150- to 135-kDa band densities, approximately 1:6-1:7 in control atrial tissues, increased to about 1:1 in atria from dogs paced for 21 days into heart failure. The density of the 150-kDa as well as that of 135-kDa eNOS bands did not change significantly in either left or right ventricular tissues. Consequently, the density ratio between these two bands in ventricular tissues after pacing was also unchanged in comparison to its value in the control group (Fig. 4.4).

DISCUSSION

This study is the first to demonstrate the enhancement of left atrial, but not ventricular, Ca^{2+}-dependent NOS activity with the induction of CHF by rapid ventricular pacing in dogs. The changes in enzyme activity were accompanied by an enhanced total eNOS protein expression, specifically the 150-kDa, but not the 135-kDa, eNOS.

The observed hemodynamic changes in dogs paced for 21 days document the occurrence of severe heart failure in these animals as previously reported in this model (2, 13, 22, 29). On the other hand, dogs paced for 2 days had some hemodynamic changes, in the absence of clinical signs of failure, suggesting the development of an early ventricular dysfunction. A unique finding in this canine model of heart failure is the development of atrial but not ventricular hypertrophy (22, 33) which is possibly caused by contraction of the atria against closed atrioventricular valves due to asynchronous electrical activation of the cardiac chambers (22, 33).

Pacing-induced heart failure caused an elevation in plasma NO_x^- levels in dogs as early as the seventh day after pacing and remained so until pacing was terminated. This occurred in the absence of any measurable renal impairment as the creatinine levels were within the normal range (70-150 μM) for this parameter in all three groups. This is in accordance with our previous report of increased plasma NO_x^- levels with age in a rat genetic model of heart failure, in which no changes in plasma creatinine were also observed (19). Therefore, enhanced levels of NO_x^- are not a consequence of renal dysfunction. In contrast, Bernstein *et al.* (8) reported in dogs paced at 210 beats per minute in the first 3 weeks then at 240 beats per minute for another week into heart failure, no changes in plasma NO_x^- within the first 3 weeks of pacing, whereas it then rose in the fourth week in direct relation to the increase in creatinine levels. They suggested that the rise in plasma NO_x^- was due to a decline in renal function, not an increase in NO production. This controversy may be related to differences in the pacing protocols and the severity of pacing-induced heart failure. Because plasma NO_x^- does not discriminate as to the tissue or organ source of the increased NO production, the changes in plasma NO_x^- levels cannot be attributed simply to enhanced atrial NOS activity. We cannot substantiate the source of increased plasma NO_x^- early in the development of heart failure (7 days) because myocardial NOS activity was not measured at this time. The contribution of NO from other sources such as the peripheral vasculature cannot be totally excluded, although a reduction in eNOS gene expression was reported in the aortic endothelium of dogs paced via the left ventricle for one month into severe CHF (37).

This report is the first to show that normal left atria have a significantly greater Ca^{2+}-dependent NOS activity than that in either left or right ventricles. We have also seen significantly greater Ca^{2+}-dependent NOS activity in the atria compared to ventricles in normal rat hearts (Chapter II). Furthermore, atrial but not ventricular Ca^{2+}-dependent NOS activity increased with pacing for 21 days and the development of heart failure. This activity measures in principle that from both eNOS and nNOS isoforms. The latter, however, has a limited distribution within nerve fibers in the heart (38) and has not been reported to contribute significantly to the cardiac Ca^{2+}-dependent NOS activity. Enhanced eNOS activity may be an effect of pacing itself since isolated adult rat ventricular myocytes show increased eNOS activity, as measured by nitrite accumulation in culture media, with increasing pacing frequency (18) or it may be caused by increased stretching (3) due to dilatation of the atria. On the other hand, the increased atrial eNOS activity in association with atrial, but not ventricular, hypertrophy suggests that the alterations in eNOS are more directly related to the changes in cardiac structure (hypertrophy) induced by the etiological factor(s) of heart failure rather than the failure state *per se*. This is supported by our data in a genetic model of heart failure in rats where left ventricular hypertrophy was accompanied by upregulation of eNOS in the same chamber (19). Upregulation of NO production in these settings may be considered a counterbalancing mechanism to the contractions of the hypertrophied atria or ventricles (19) since NO is known to have negative inotropic effects in the normal heart (5, 6, 9).

Although our data is in accordance with other reports showing an enhanced endothelium-dependent relaxation response and NO production in the coronary arteries of dogs with pacing-induced CHF (20, 25), they contrast the evidence for a diminished coronary and peripheral vascular NO generation reported by other investigators (37, 41). This controversy may be related to the severity of CHF in any particular study as determined by the rate or the duration of pacing. Moreover, these investigators utilized isolated coronary vascular rings (20, 25) or studied the whole coronary circulation *in situ* (41) as compared to our findings in the left atria tissues. On the other hand, these data may suggest a differential regulation of NO generation in the coronary and peripheral vascular tree from that in atrial tissue in CHF induced by ventricular pacing. As the endocardium was removed from all heart samples we can exclude its contribution to atrial NOS activity.

Previous studies in humans addressed the hypothesis that NO elaborated by the induction of Ca^{2+}-independent NOS (iNOS) is a potential factor associated with the development of CHF. They reported that the expression of iNOS mRNA and/or protein takes place in ventricular but not atrial tissues, and in patients with a variety of causes of failure (11, 12, 16, 40). Contrary to our expectation, Ca^{2+}-independent NOS (iNOS) activity was not detectable in atria and ventricles from control or paced dogs in this study. This is in accordance with a recent report from this laboratory whereby iNOS activity was also not detected in ventricles from heart failure-prone rats (19). These findings cannot be explained by the insensitivity of our NOS assay since we were able to detect Ca^{2+}-independent NOS activity in hearts from rats treated with bacterial endotoxin (19). In comparison with human studies, the lack of iNOS expression in this study or the rat genetic model of heart failure (19) may be related to species variations or because of differences in the etiologies of heart failure. Whether the upregulation of eNOS in dogs with heart failure is cardioprotective through vasodilatory, anti-aggregatory and negative chronotropic actions of NO or detrimental in a manner usually ascribed to the expression of iNOS in the heart whereby enhanced NO production, in addition to its negative inotropic effects (4-6, 9, 30, 39), may induce myocyte death through cytolysis (28) or apoptosis (24), is unknown.

We have detected in the atria by Western blot analysis using a polyclonal eNOS antibody the presence of three bands corresponding to the known molecular weight of eNOS, whereas only two bands were found in the ventricles. Previous work demonstrated doublet bands corresponding to the 150- and 135-kDa proteins in adult rat ventricular myocytes (7) and in COS-7 cells that had been transfected with wild-type eNOS or myristoylation-deficient eNOS mutant cDNAs (10) using 7% SDS-PAGE. Unlike these studies (7, 10) in our hands multiple atrial and ventricular bands in dogs were also detected by 9% SDS-PAGE. At the present time, we do not know the nature of the third (lowest) band detected in the atrial samples but it is unlikely that it represents a degradation product since atrial and ventricular tissues were harvested from dogs at the same time, processed and analyzed simultaneously. It may be related to another post-translational modification of eNOS in the atria, for example glycosylation that may not take place in the ventricles.

Endothelial NOS is synthesized as a 150-kDa protein (7) which undergoes post-translational modification before it is targeted to sarcolemmal caveolae in a process that involves myristoylation (10, 34, 36) and palmitoylation (21, 36) as a 135-kDa protein. Our data indicates that pacing induced-heart failure increases the absolute abundance of 150-kDa eNOS as well as its expression relative to that of the 135-kDa. Because total eNOS and 150-kDa expression were enhanced with no changes in the expression of the 135-kDa protein, it is possible that these changes are related to increased eNOS mRNA synthesis in association with insufficient intracellular mechanisms for the processing and trafficking of eNOS to the plasmalemmal caveolea. Interestingly, these alterations in the expression of 150-kDa eNOS were accompanied by increased enzyme activity, despite findings suggesting that post-translational processing and targeting of eNOS into the plasmalemmal caveolae are necessary for its optimal activity (10, 21, 34, 36). In a study of cultured adult rat cardiomyocytes, Belhassen *et al.* reported that even a short term elevation in intracellular cAMP inhibits the post-translational processing and the translocation of 150-kDa eNOS (7). However, either basal cAMP production or that stimulated by forskolin or isoproterenol is reduced in the ventricles of dogs paced into CHF (17, 27). If this is also the case for cAMP levels in atrial myocytes from CHF dogs, then the increase in the atrial 150-kDa eNOS seen in this study cannot be explained by the same mechanism.

Several limitations of this study warrant further discussion. This method of pacing-induced CHF in dogs may not model various types of human heart failure that develop as a result of ischemic or valvular heart diseases or inflammatory cardiomyopathies but it does mimic ventricular dysfunction and cardiomegaly seen in patients with chronic uncontrolled tachycardia (26). Moreover, there are clear differences between dogs and humans with respect to age and the rate of development of CHF in addition to the possible species variations. We did not investigate right atrial tissues in this study as they were used in a different study and tissue sampling was limited to one site in each of the three chambers studied. There are, however, no previous data to support the possibility of differences in the activity and/or expression of NOS between and/or within heart chambers. In addition, we do not know the cellular source of enhanced eNOS expression in the left atria, whether this occurs in coronary endothelial cells and/or cardiac myocytes is unknown and would require immunohistochemical localization. Despite these limitations, our data stress the need to carefully examine the role of eNOS in the setting of human heart failure and hypertrophy, which has been overlooked. It may be of particular interest to investigate this in patients with mitral stenosis which causes left atrial but not left ventricular hypertrophy.

We have shown that increased left atrial, but not ventricular, Ca^{2+}-dependent NOS activity along with enhanced expression of a 150-kDa eNOS occurs *in vivo* with rapid ventricular pacing-induced heart failure in dogs. Further investigation is necessary to reveal the consequences of these alterations in eNOS activity and expression in regards to the possible cardioprotective or detrimental roles of NO in the myocardium.

Table 4.1 Hemodynamic parameters before (Pre-pacing) and after 2 or 21 days of rapid ventricular pacing in dogs.

		pacing at 250 bpm	
	Pre-pacing (12)	**2 days (5)**	**21 days (7)**
LV systolic pressure (mmHg)	139 ± 23	3 ± 22	106 ± 11*
LV end diastolic pressure (mmHg)	8 ± 4	13 ± 4	31 ± 9*
LV dP/dt (mmHg/sec)	2778 ± 316	1755 ± 141*	1474 ± 253*
Mean arterial pressure (mmHg)	111 ± 6.2	93.2 ± 4	87.6 ± 2.7*
Right atrial pressure (mmHg)	1.8 ± 0.5	4.9 ± 1.1	7.5 ± 1.4*
PCWP (mmHg)	3.1 ± 0.6	11.1 ± 1.2	19.7 ± 4.7*
Heart rate (sinus rhythm, bpm)	93 ± 4	121 ± 8*	125 ± 9*
Cardiac output (ml/min)	2671 ± 215	2209 ± 251	1518 ± 113*
Ejection Fraction (%)	55 ± 1	44 ± 2*	25 ± 2*

LV ejection fraction was determined by echocardiography. Values are means ± SE for n dogs as given in brackets. LV, left ventricular; PCWP, pulmonary capillary wedge pressure; * $p < 0.05$ versus Pre-pacing.

Table 4.2 Changes in left atrial and ventricular weights and cross-sectional area before (Pre-pacing) and after 2, 7 or 21 days of rapid ventricular pacing in dogs.

		pacing at 250 bpm		
	Pre-pacing (12)	**2 days (5)**	**7 days (7)**	**21 days (7)**
Weights (g)				
left atrial appendage	1.8 ± 0.2 (8)	1.9 ± 0.2		3.7 ± 0.6*†
left ventricle	96 ± 4	112 ± 5	101 ± 6	104 ± 10
heart	177 ± 14 (8)	180 ± 7		179 ± 13
left atrial appendage/heart	0.009 ± .003	0.010 ± .002		0.020 ± .005*†
Cross-sectional area (cm²)				
left atrium	8.3 ± 0.3	10.6 ± 0.4*	12.7 ± 0.6*†	15.2 ± 0.6*†
left ventricle	14.4 ± 0.6	14.7 ± 0.8	19.2 ± 0.9*†	21.4 ± 1.4*†

Left ventricle weight and left atrial and ventricular cross-sectional areas were determined by echocardiography. Other weights were obtained by weighing tissues collected at the time of sacrifice. The left atrial appendage and total heart weights under "Pre-pacing" were obtained from the non-paced control group (n = 8). Values are means ± SE for n dogs as given in brackets. * $p < 0.05$ versus Pre-pacing or control, †$P < 0.05$ versus 2 days.

Fig. 4.1 Changes in plasma nitrate/nitrite (NO_x^-, panel A) and creatinine (panel B) levels with rapid ventricular pacing in dogs. Blood samples were taken before pacing (pre-pacing) and at 7 and 21 days of pacing into heart failure. Another group of dogs was paced for 2 days and sampled before and at the end of pacing. The number in each column refers to the number of dogs per group. Values are means ± SE. * $P < 0.05$ versus pre-pacing and 2-day levels.

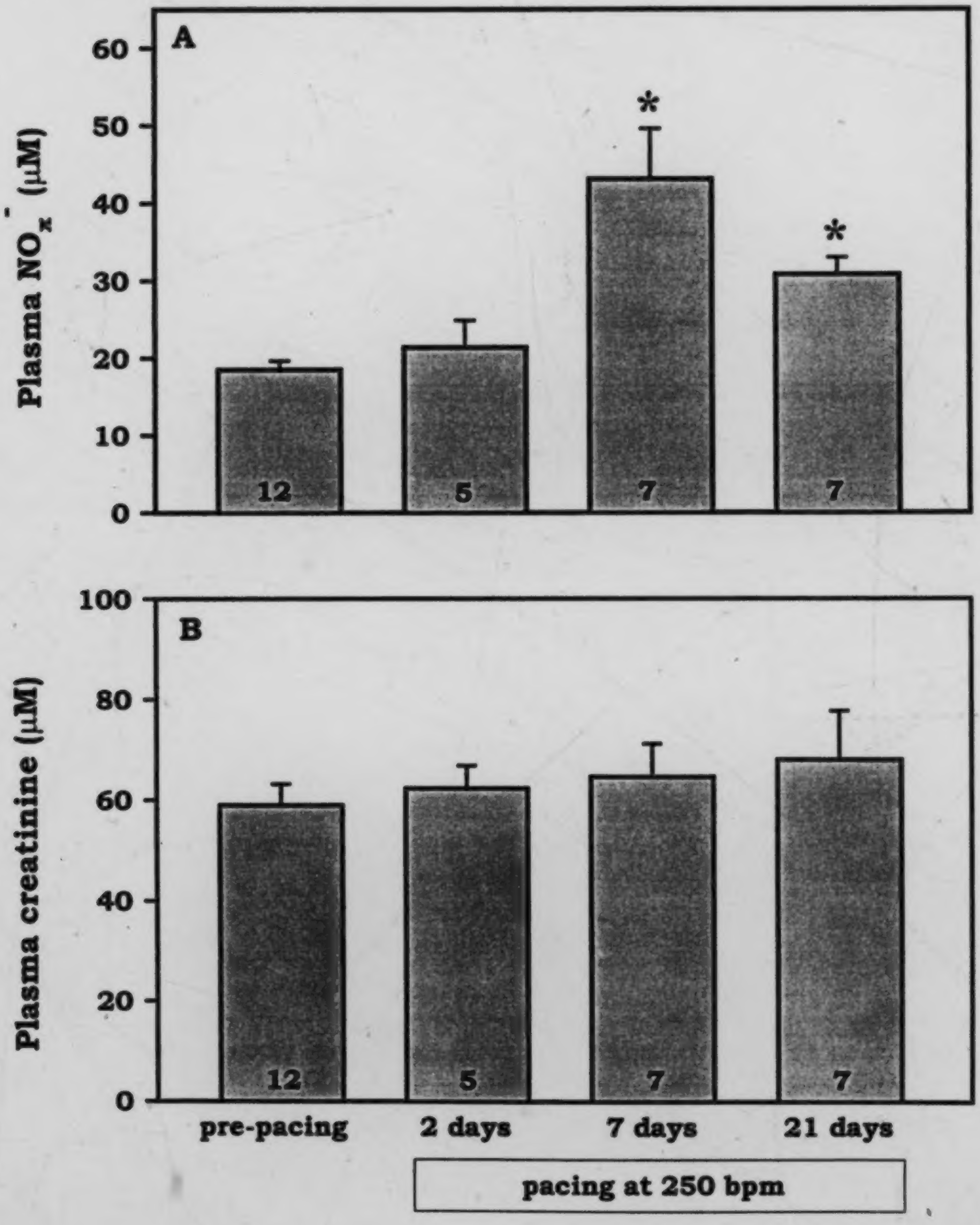


Fig. 4.2 Changes in atrial and ventricular Ca^{2+}-dependent NOS activities with rapid ventricular pacing in dogs. Tissues from non-paced controls, 2-day paced dogs and dogs paced for 21 days into heart failure were assayed. . The number in each column refers to the number of dogs per group. Values are means ± SE. * $P < 0.05$ versus atria from control or 2-day paced dogs, † $P < 0.05$ versus left and right ventricle values within the same group of dog

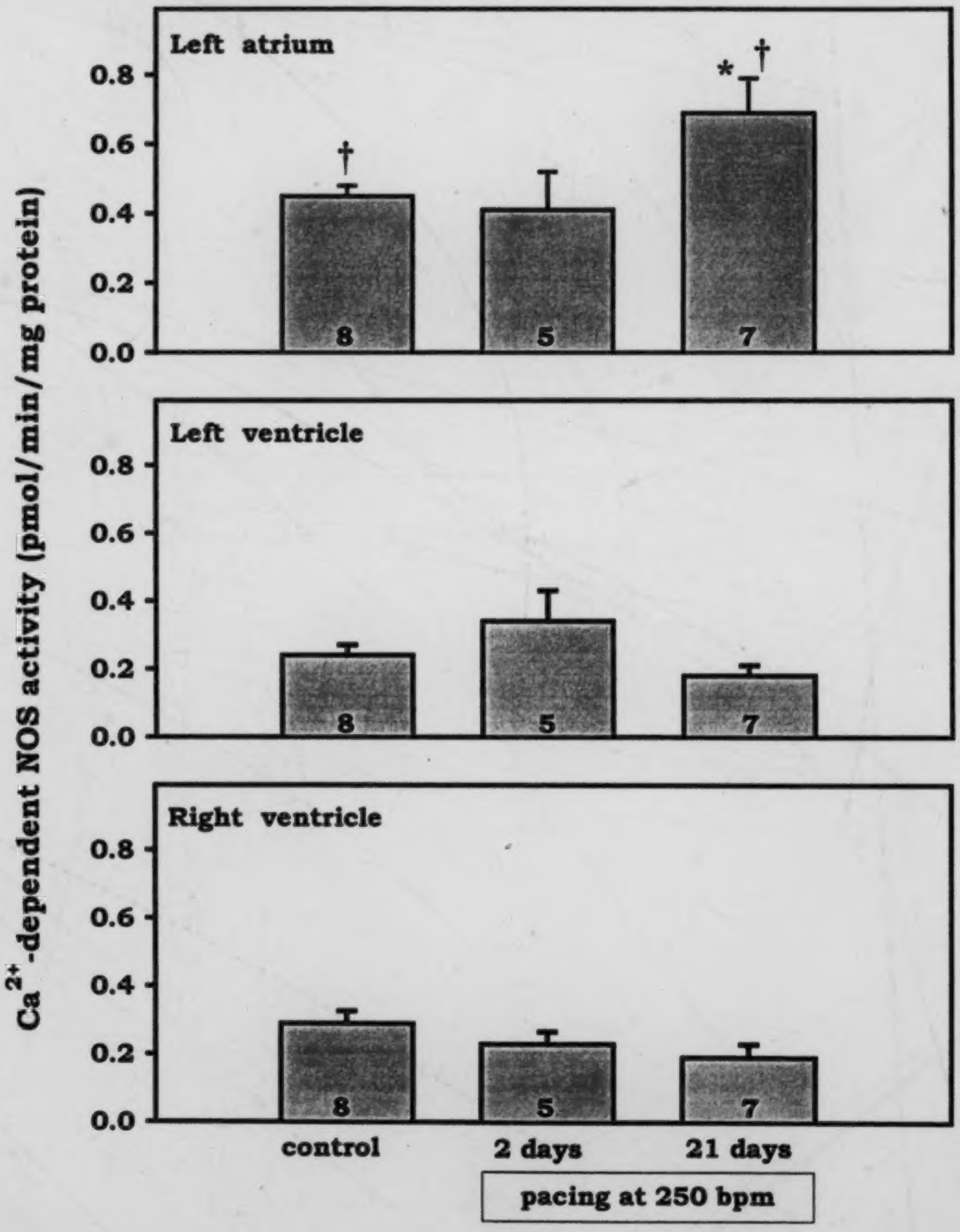


Fig. 4.3 Representative blots of immunoreactive endothelial NOS (eNOS) proteins in the left atria and ventricles of dogs. Each blot is a tissue sample taken from one dog. Tissues from non-paced controls (n = 8), 2-day paced dogs (n = 5) and dogs paced for 21 days into heart failure (n = 7) were analyzed. 50 µg protein were loaded per lane and were size-fractionated by 7% SDS-PAGE. LA, left atrium; LV left ventricle; RV right ventricle.

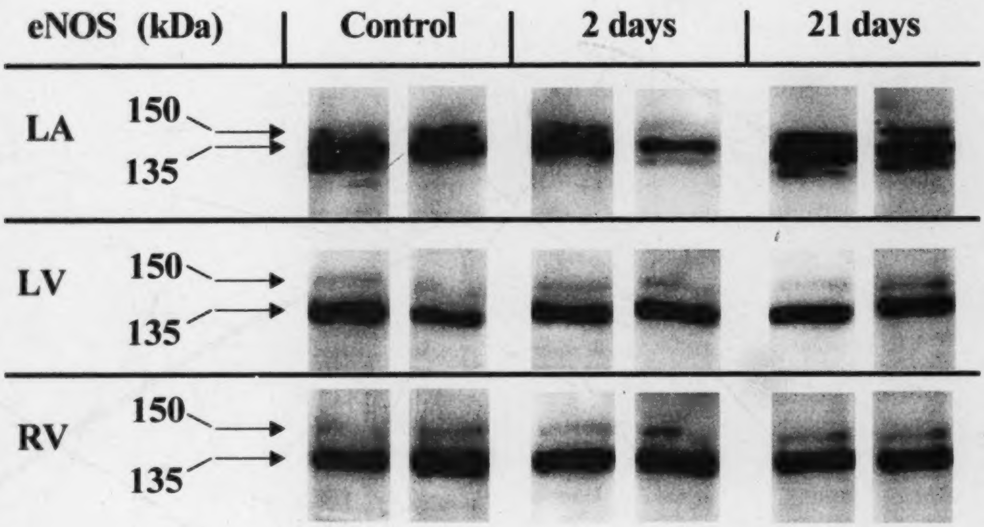
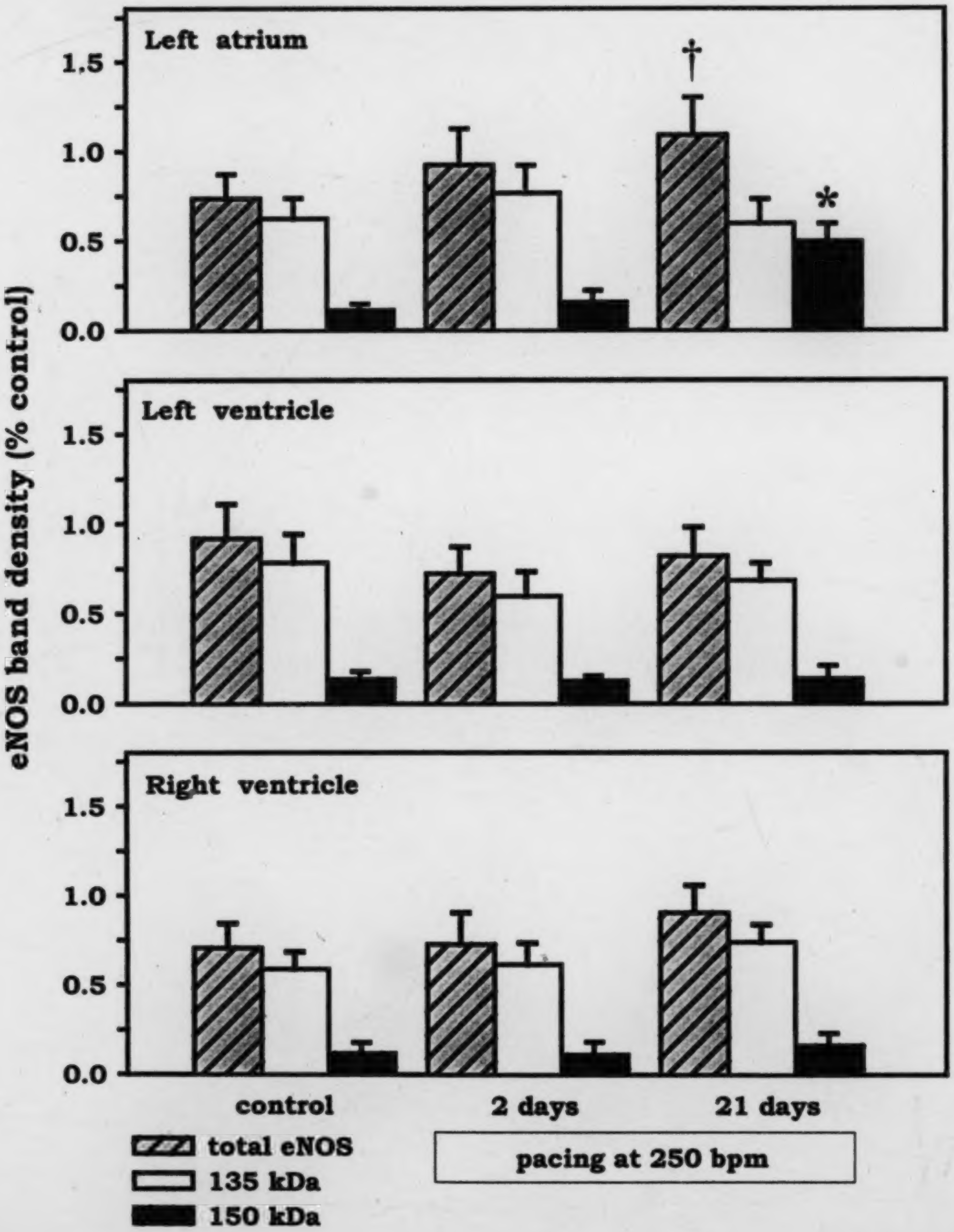

eNOS (kDa)
Control
2 days
21 days
LA
150
135
LV
150
135
RV
150
135

Fig. 4.4 Densitometric analysis of endothelial NOS (eNOS) protein band expression in atrial and ventricular tissues from dogs. Tissues from non-paced controls (n = 8), 2-day paced dogs (n = 5) and dogs paced for 21 days into heart failure (n = 7) were analyzed. Total eNOS, density of both 150- and 135-kDa bands. Values are means ± SE. * $P < 0.05$ versus control and 2-day 150-kDa bands, † $P < 0.05$ versus control total eNOS.



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CHAPTER V

AGE-DEPENDENT AUGMENTATION OF CARDIAC ENDOTHELIAL NITRIC OXIDE SYNTHASE IN A GENETIC RAT MODEL OF HEART FAILURE

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Data in Tables 5.1 & Fig. 5.1 are by RHK & SP and in Fig. 5.2A by Ken Strynadka.

INTRODUCTION

Nitric oxide (NO) is synthesized by an oxidoreductase called the nitric oxide synthase (NOS). To date three isoforms of NOS have been identified. Neuronal NOS (nNOS, NOS I) and endothelial NOS (eNOS, NOS III) are constitutively expressed and calcium-calmodulin-dependent isoforms. In contrast, the activity of NOS II (iNOS), whose expression is induced by immunological stimuli, is Ca^{2+}-independent (18).

NO, synthesized continuously in the endothelium by eNOS, regulates both vascular tone and blood pressure through its vasodilator action (18). Along with coronary vascular endothelium (1), the endocardial endothelium (24) and cardiac myocytes (4, 25) constitutively express eNOS in the normal heart. Cardiac NO production is known to play physiological roles in regulating coronary blood flow (1) and modulating contractile function (27) by exerting negative inotropic (2, 4, 5) and chronotropic (2, 11) actions. Upregulation of cardiac NO biosynthesis, as a consequence of the expression of iNOS following exposure of the heart to pro-inflammatory cytokines or bacterial endotoxin, causes a pathophysiological depression in myocardial contractile function (3, 26, 30) and possibly a direct cytolytic effect on cardiac myocytes (22).

There is now considerable evidence that the production of NO is abnormal in heart failure. However, the cause of the abnormality and the role of NO in the pathogenesis of this disorder are unclear. Both an increase (21) and decrease (20) in vascular NO production with impaired (32) or enhanced (16) endothelium-dependent relaxations have been shown in experimental heart failure. Measurements of cardiac NOS in human congestive heart failure have revealed conflicting results. DeBelder *et al.* reported a Ca^{2+}-independent NOS activity in the right ventricular myocardium of patients with idiopathic dilated cardiomyopathy (6). Haywood *et al.* found expression of iNOS mRNA and protein in ventricular myocardium from patients with heart failure due to ischemic and valvular heart disease and dilated cardiomyopathy (13) whereas iNOS protein expression was detected by Thoenes *et al.* in failing hearts from patients with septic shock only (31). In spite of these findings, measurements of cardiac NOS activity and expression during the development of heart failure have not been reported.

The objective of the present study was to investigate the age-dependent changes in cardiac NO production in a genetically determined heart failure model. We measured ventricular NOS activity and expression, as well as the level of plasma nitrate and nitrite (NO_x^-) in this unique rat model of spontaneous and reproducible congestive heart failure, the heart failure-prone (SHHF/Mcc-*fa*^{cp}) rat (7, 12, 17). SHHF rats are spontaneously hypertensive animals that initially show hypertrophy of the left ventricle, followed by right ventricular hypertrophy with dilated cardiomyopathy which eventually progresses to heart failure from approximately 14 months of age and onwards (7, 12, 17). For comparison we also examined age-matched Spontaneously Hypertensive rats (SHR), which are hypertensive like SHHF rats, but do not develop heart failure and normotensive Sprague Dawley (SD) rats.

MATERIALS AND METHODS

Experimental design

At the onset of the study, male rats from 3 different strains (SD, SHR and SHHF) were randomly allocated into three age group categories (6, 12 and 18 months), with six animals included per group. SD rats and SHR rats were obtained from Harlan Sprague Dawley, Inc., (Indianapolis, IN) at approximately 5 months of age. Phenotypically lean male SHHF rats were obtained from Dr. Sylvia McCune (Ohio State University, Columbus, OH). All animals were allowed food and water *ad libitum* and maintained on 12 hr light-dark cycles. Lean male SHHF rats are known to develop heart failure from approximately 14 months of age and onwards (12, 17). Therefore, as we wished to determine whether there are changes in NO production during the progression to heart failure, animals were sacrificed at either 6, 12 or 18 months of age.

Experimental procedures

Immediately after light anesthesia with intraperitoneal ketamine/xylazine (10 mg/kg and 50 mg/kg, respectively), systolic blood pressure was measured using an IITC tail cuff pump (Woodland Hills, CA) attached to a Gilson Duograph (GILSON Medical Electronics, Middleton, WI). This measurement was done at least 3 days prior to sacrifice. On the day of sacrifice (between 9 a.m. and 12 noon) rats were weighed, then pentobarbital sodium (100 mg/kg, i.p.) was injected and the thoracic and abdominal cavities were exposed immediately upon attainment of anesthesia. Blood was collected by cardiac puncture into the left ventricular apex. One milliliter of blood was immediately placed in a 1.5 ml microcentrifuge tube containing 20 μl heparin sodium (1000 units/ml, Elkins-Sinn, Cherry Hill, NJ). The tube was inverted three times and centrifuged at 2000 xg for 4 min at 4°C. Plasma was separated and stored at -80°C until analysis. Another milliliter of blood was allowed to clot in another tube for serum collection. Meanwhile, hearts were rapidly excised and perfused through the aortic cannula with 50 ml of Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) at room temperature, supplemented with 2.45 g/L sodium bicarbonate and 1% penicillin/streptomycin, to wash them free of blood. The right ventricle and left ventricle with the interventricular septum were dissected, weighed, and then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Measurements of plasma NO_x^- and serum creatinine

After thawing, plasma was diluted 1:1 with deionized water. Then 400 μl of the diluted plasma was deproteinized by centrifugal ultrafiltration (Ultrafree-MC microcentrifuge tubes UFC3, Millipore, MA, USA). Ultrafiltrates were analyzed for total nitrate and nitrite (NO_x^-) content by the method of Green *et al.* (9). The limit of detection was 0.1 μM nitrite or nitrate. Renal function was assessed through determination of serum creatinine levels by a colorimetric method based on Jaffe's reaction utilizing a Sigma diagnostics creatinine kit.

Preparation of ventricular homogenates for determination of NOS activities

The frozen ventricular tissue was crushed in a pestle and mortar cooled to the temperature of liquid nitrogen. The powder was placed in five volumes of ice-cold homogenization buffer [composition given in Schulz *et al.* (25)] and homogenized with an Ultra-Turrex disperser using three strokes of 3 sec duration each. The homogenate was centrifuged (1000 xg for 5 min) at 4°C and the supernatant was kept on ice for immediate assay of NOS activities. NOS activities in left ventricular homogenates was measured by the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline according to Schulz *et al.* (26). Samples were incubated for 13 min at 37°C in the presence or absence of either EGTA (1 mM) or EGTA plus L-NMMA (1 mM) to determine the level of Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities, respectively. Citrulline production was normalized to the protein concentration of the homogenates, as determined by bicinchoninic acid assay utilizing bovine serum albumin as a standard. NOS activity was expressed as pmol/min/mg protein and the limit of detection was 0.1 pmol/min/mg protein.

As a positive control for the detection of Ca^{2+}-independent NO synthase activity in the heart a separate series of four Sprague Dawley rats (250-300 g) were injected with *Salmonella typhosa* lipopolysaccharide (4 mg/kg, i.p., Difco laboratories, Detroit, MI) prepared in pyrogen-free saline and another four received vehicle, as previously described (25). Six hours later the animals were sacrificed with pentobarbital sodium (100 mg/kg, i.p.) and a segment of the left ventricular wall was excised, rapidly frozen in liquid nitrogen and processed for determination of Ca^{2+}-independent NO synthase activity as above.

Western blotting and densitometric analysis

After adjusting the protein concentration in the crude supernatant of samples to 3 μg/μl with homogenizing buffer, proteins were denatured by boiling for 5 min in the presence of sodium dodecyl sulfate (SDS, 6%) and 3% mercaptoethanol. 90 μg of the denatured proteins were size-fractionated by polyacrylamide (9%) gel electrophoresis and transferred to nitrocellulose membranes (Trans-blot transfer medium, Bio-Rad) by wet electroblotting for 2 hours using an electrophoretic transfer cell (Bio-Rad). Prestained SDS-PAGE protein standards (Bio-Rad) were used as molecular weight markers. The membranes were blocked at room temperature in 10% skimmed dried milk in phosphate-buffered saline (PBS) for 3 hr, thereafter they were incubated with murine monoclonal antibodies directed against human eNOS (Transduction Laboratories, Lexington, KY; prepared in 1% skimmed milk in PBS at 1:4000 dilution) for 3 hr, then washed twice with PBS with 0.05% Tween-20 (5 min each) and finally with PBS alone. Subsequently, the membranes were incubated for 60 min with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Transduction Laboratories) diluted 1:2000 with 1% skimmed milk in PBS. The membranes were washed four times (5 min each) in PBS containing 0.05% Tween-20, followed by 3 washes (5 min each) in PBS alone. The immunoreactive proteins were detected by using an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (ECL, Amersham) according to the manufacturer's instructions. Autoradiographs were obtained by exposure to X-ray film (Kodak, X-OMAT) for 45-75 sec and the density of the bands were quantified by scanning densitometry using NIH Image computer software (Wayne Rasband, RSB, NIMH, NIH Bethesda, MD) calibrated using a Kodak optical density gray scale and expressed in percent relative to the control. Under these conditions, signal intensities were proportional to the amount of the total protein loaded and within the linear range of the film. As an external control for each blot, we utilized a single heart sample obtained from an eight week old SD rat which had been isolated and processed as above.

Materials

All compounds were purchased from Sigma except L-[U-^{14}C]-arginine monohydrochloride (Amersham) and L-NMMA (a kind gift from the Wellcome Research Laboratories, Beckenham, U.K).

Statistical analyses

Data are presented as means ± SE. Values of n refer to the number of rats in each group. A two way analysis of variance followed by Tukey's post hoc test was used to evaluate differences between the groups. Linear regression analysis was utilized to examine for correlation between parameters. Changes in blood pressure with age were analyzed by one way ANOVA with the Tukey-Kramer multiple comparisons test. Data analysis was done using a statistical software package (SigmaStat, version 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at $P < 0.05$.

RESULTS

At 6 and 12 months of age, none of the SHHF rats exhibited any overt signs of congestive heart failure. At 18 months of age, one of the SHHF rats showed signs of labored breathing, piloerection and cachexia. Upon sacrifice fluid was found in the chest cavity and a large thrombus was observed in the left atrium. Another animal from the same group had to be sacrificed at 17 months of age because of severe peripheral edema, and a large left atrial thrombus was also observed at sacrifice. The other animals within this group showed no overt clinical signs of heart failure either before sacrifice or at autopsy; one animal showed left atrial thrombi only. At all age groups, SD and SHR rats showed no signs of heart failure.

Ventricular weights and blood pressure

The blood pressure and left ventricular weight data are depicted in Fig. 5.1. Blood pressure in SHHF and SHR rats (mean of all three age groups was 185 ± 4 and 183 ± 3 mmHg, respectively) was significantly higher than that in SD rats (125 ± 6 mmHg). No significant changes in blood pressure with age were noted in SD rats or the hypertensive SHR rats (Fig. 5.1A). In contrast, there was a significant decline in blood pressure of SHHF rats with increasing age so that 18 month old animals had a lower blood pressure than those at 6 and 12 months (Fig. 5.1A). The left ventricular weights in SHHF and SHR rats were not significantly different (mean of all three age groups was 1.34 ± 0.04 and 1.40 ± 0.03 g, respectively), but both were significantly higher than that of SD animals (1.13 ± 0.03 g, $p < 0.05$). Eighteen-month-old SHHF and SHR rats as well as 12 month old SHR rats had higher left ventricular weights than 6 month old animals in the same strain (Fig. 5.1B). Moreover, at 18 months of age, both SHHF and SHR rats had a significantly higher left ventricular weight than SD animals.

Table 5.1 shows body weight, right ventricular weight, and right as well as left ventricular weight/body weight ratios in the three strains of rats determined at 6, 12 and 18 months of age. There were no statistically significant differences in body weight, right ventricular weight or its ratio to body weight between the three rat strains at any age. The ratio of left ventricular weight to body weight was significantly increased at 18 months in SHHF rats compared to both 6 and 12 months. In SHR rats, this ratio was already significantly increased at 12 months relative to 6 months, with no further changes at 18 months. No significant changes in this ratio were observed with age in SD rats.

Plasma NO_x^- level

The plasma levels of NO_x^- are represented in Fig. 5.2A. In both SHHF and SHR rats, plasma NO_x^- increased at 18 months in comparison to that at 6 and 12 months of age but it did not significantly change with age in SD rats. As a result, plasma NO_x^- in 18-month-old SHHF and SHR rats was 2.2 fold and 1.8 fold higher than that of the age-matched SD animals, respectively.

Serum creatinine level

Serum creatinine levels were within the normal range of 0.2-0.8 mg/dL in the three strains of rats at any age group with no significant differences between them (mean of all three age groups was 0.58 ± 0.03, 0.52 ± 0.03 and 0.64 ± 0.03 mg/dL for SHHF, SHR and SD rats, respectively).

Left ventricular NOS activities

Ca^{2+}-dependent NOS activity in left ventricular homogenates is depicted in Fig. 5.2B. In SHHF rats, this activity was markedly elevated by 18 months of age in comparison to that observed at 6 and 12 months of age. The enzyme activity was significantly higher in 18 month old SHR rats also but only in comparison to 6- month-old animals. In SD rats, there were no significant differences in the Ca^{2+}-dependent NOS activity between various age groups. Accordingly, the enzyme activity in 18-month-old SHHF rats was significantly higher than that in age-matched SHR and SD animals. Ca^{2+}-independent NOS activity was not detectable in any of these hearts (data not shown).

In contrast, the iNOS positive control, lipopolysaccharide-treated rats showed a high ventricular Ca^{2+}-independent NOS activity (2.22 ± 0.20 pmol/min/mg protein, n=4) compared to vehicle-injected rats (0.13 ± 0.03 pmol/min/mg protein, $p < 0.0001$, n=4).

Western blot and densitometric analysis

Endothelial NOS was detected in all ventricular homogenates by Western blot analysis using an eNOS specific antibody (Fig. 5.3). A 135-kDa band was observed, corresponding to the known molecular weight of eNOS (23). Enhanced expression of eNOS was seen in the hearts of SHHF rats at 18 months of age compared to that in age-matched SHR and SD rats, (Fig. 5.3A). Densitometric analysis of eNOS expression in all strains and age groups is shown in Fig. 5.3B. In marked contrast to SHR and SD rats, SHHF rats showed an age-dependent increase of eNOS expression so that by 12 months these animals had a 1.7 fold higher enzyme expression than that of 6-month-old rats and expressed a significantly more eNOS protein than age-matched SHR and SD rats. By 18 months, eNOS expression in SHHF rats was 3.5 fold and twofold higher than that at 6 and 12 months of age, respectively. Moreover, eNOS protein level in 18-month-old SHHF rats was about threefold higher than that in age-matched SHR and SD rats. No changes in eNOS band density with age were seen in SHR and SD rats.

Correlational analysis

In SHHF rats only, there was a statistically significant linear correlation between cardiac Ca^{2+}-dependent NOS activity and plasma NO_x^- levels (Fig. 5.4A). However, the changes in plasma NO_x^- did not correlate with serum creatinine levels in these animals (data not shown). In addition, the increase in plasma NO_x^- and Ca^{2+}-dependent NOS activity in SHHF rats correlated significantly to the increase in left ventricular weight (r=0.563, p=0.015 and r=0.719, p=0.0008, respectively, data not shown). Moreover, there was a strong negative correlation between the increase in Ca^{2+}-dependent NOS activity and the age-dependent decline of blood pressure in SHHF rats (Fig. 5.4A). Cardiac eNOS protein expression was positively correlated with both plasma NO_x^- levels and Ca^{2+}-dependent NOS activity (Fig. 5.4B) in SHHF rats. In marked contrast to the SHHF rat, the correlations between all of these different parameters were not statistically significant for either SHR or SD rats (data not shown).

DISCUSSION

The present study for the first time demonstrates an age-dependent increase in cardiac NOS in the spontaneously hypertensive and genetically heart failure prone SHHF rat as detected by the enhanced Ca^{2+}-dependent NOS activity and eNOS expression in left ventricular homogenates, along with increased plasma NO_x^- levels. Although both SHHF and SHR rats were hypertensive and showed an increase in left ventricular mass over time, only in SHHF rats did the changes in blood pressure and left ventricular weight correlate with the alterations in ventricular NOS activity, eNOS expression and plasma NO_x^- levels.

Blood pressure and left ventricular weight data are in agreement with the previously published reports regarding the hemodynamic changes that occur in SHHF rats with age (7, 12, 17). Our results showed a statistically significant increase in left ventricular mass in these hypertensive animals only at 18 months. Although there was a 50% increase in right ventricular mass in SHHF rats (18 vs. 6-month-old animals), this did not reach statistical significance most probably due to the small number of animals included in this study. It is also possible that right ventricular hypertrophy was not detected because not all of the SHHF rats were in failure by 18 months of age. But it can be inferred that these hypertensive animals had a decompensating heart from the age-dependent decline of blood pressure. Although humoral indicators of heart failure were not measured in this study, a corresponding study reported that all 18 month old SHHF rats have marked elevations in plasma renin activity, atrial natriuretic peptide and aldosterone compared to age-matched SHR and SD rats (14). On the other hand, SHR rats rarely manifest clinical signs of heart failure before old age (20 to 22 months), even though all animals develop progressive left ventricular hypertrophy in the setting of persistently elevated blood pressure (12). Studying SHHF rats older than 18 months, or considering severe congestive heart failure as the end point at which animals are sacrificed, may be necessary to show all of the previously reported changes in cardiac and non-cardiac parameters indicative of the development of heart failure. We also understand that the body weight in heart failure represents a composite of fluid retention and loss of lean body tissue, therefore, the ratios of left and right ventricular weights to body weight in SHHF rats are not accurate indicators of ventricular hypertrophy.

The marked age-dependent increase in Ca^{2+}-dependent NOS activity with enhanced eNOS expression in ventricular tissues from SHHF rats indicates an upregulated cardiac NO production during the onset or early stages of the progression to heart failure. Unlike SHHF rats, Ca^{2+}-dependent NOS activity was only modestly elevated in SHR rats by 18 months of age and was not accompanied by a change in eNOS expression. In fact, an elevated Ca^{2+}-dependent NOS activity in cardiac endothelial cells isolated from SHR rats was reported by Nava *et al.* at 4.5-6 months of age, compared to one month old animals (19). This differential enhancement in the activity and expression of eNOS in SHHF rats from that in SHR rats suggests a possible relationship between increased cardiac NO production and a progression to heart failure. Since NO is known to exert a negative inotropic effect (2-5, 26, 30), our findings therefore point to a possible role for NO in contractile dysfunction of the failing heart.

Surprisingly, there was no detectable Ca^{2+}-independent NOS (iNOS) activity in hearts from any of the SHHF rats. This is despite previous studies which documented the expression of iNOS protein and/or mRNA in ventricular myocardium samples from heart failure patients (13, 31) and the co-localized expression of iNOS and tumor necrosis factor-α in cardiac tissues from end-stage congestive heart failure patients with dilated cardiomyopathy (10). These studies (10, 13, 31), however, did not investigate eNOS expression and also did not determine NOS activities in parallel to their findings with iNOS mRNA and/or protein expression. Our results cannot be explained by the insensitivity of our assay in detecting cardiac iNOS activity since we were able to measure a high Ca^{2+}-independent NOS activity in lipopolysaccharide-treated rats, as we have previously found (25). Based on our present findings and these other studies (with the expressed limitations of possible species differences and differences between this genetic model and the multifactorial nature of human heart failure) we speculate that eNOS may be upregulated either before or near to the development of heart failure. It could then be downregulated during end stage, severe failure where induction of iNOS may occur through the action of inflammatory cytokines which are known to stimulate cardiac iNOS expression (3, 25, 26), as well as reduce the expression of eNOS by destabilizing its mRNA (35). In support of this, dogs with pacing-induced heart failure were reported to have a diminished gene expression of vascular eNOS (28) along with a defective endothelium-mediated control of the coronary circulation (32).

The plasma level of nitrate/nitrite (NO_x^-) has been used extensively as an index of NO production (25, 33, 34, 36) because it represents the stable end products of endogenous NO metabolism and are readily measured, unlike NO itself. In heart failure patients, Winlaw and co-workers were the first to report elevated plasma nitrate levels compared to healthy individuals (33). Subsequently, these investigators showed that this elevation occurred in proportion to the severity of heart failure (34). It is clear that plasma NO_x^- does not discriminate as to the tissue or organ source of increased NO production. However, a number of studies have indicated a net production of NO by the heart. For example, Zeballos *et al.* showed a positive veno-arterial nitrate difference in the coronary circulation of resting conscious dogs, but not across the pulmonary or skeletal muscle beds (36). Kelm and Schrader have demonstrated a continuous release of NO from the isolated heart (15). These reports and the observed correlations in this study between plasma NO_x^- levels, cardiac Ca^{2+}-dependent NOS activity and ventricular eNOS expression in SHHF rats, suggests that the heart is a prominent contributor to the elevated plasma NO_x^- via enhanced NO production. Some of the plasma NO_x^- also originates from the peripheral vasculature eNOS activity, but we do not know its relative contribution. As serum creatinine levels in these rats were normal this excludes renal dysfunction, and therefore impaired NO_x^- excretion, as a possible cause of elevated plasma NO_x^-. This conclusion is further reinforced by the lack of correlation between serum creatinine and plasma NO_x^- levels in SHHF rats.

The level of plasma NO_x^- was also elevated in SHR rats, but unlike SHHF rats, it neither correlated with Ca^{2+}-dependent NOS activity nor eNOS expression, nor to changes in blood pressure or left ventricular weight. Plasma NO_x^- also did not correlate to serum creatinine in these rats. It is possible, therefore, that the increase in plasma NO_x^- in SHR rats was due to enhanced

NO production by the peripheral vascular endothelium in response to an elevation of blood pressure as well as NO formed by cardiac tissue (19).

This study was not specifically designed to address the role of NO in the pathophysiology of congestive heart failure. It is possible that the elevated NO production in hearts of SHHF rats results in contractile dysfunction (3, 5, 30) or myocyte death through cytolysis (22) or apoptosis (8). On the other hand, it may play a cardioprotective role in this setting through its vasodilator and antiaggregatory properties. The cellular localization of eNOS was not determined in this study but it has been reported to exist in coronary and endocardial endothelia (1, 24) as well as in cardiac myocytes (4, 25). For this reason it is difficult to predict the mechanism by which eNOS was upregulated in the hearts of SHHF rats. We also cannot exclude the possibility that neuronal NOS, the other Ca^{2+}-dependent NOS which has been localized to some cardiac neurons (29), might have contributed to the changes in NOS activity observed, although this is rather unlikely considering that neurons comprise only a minor fraction of total heart weight.

The implications from this work regarding the relationship between an enhanced cardiac NO production and the development of congestive heart failure, require further study. The SHHF rat represents a unique genetic model of heart failure which should provide further insights into these questions.

Table 5.1 Body weight, left ventricular and right ventricular weights and right ventricular weight/body weight ratio in Sprague-Dawley, Spontaneously Hypertensive and Heart Failure-prone rats at 6, 12 and 18 months of age.

	Sprague Dawley rats			**Spontaneously Hypertensive rats**			**Heart Failure-prone rats**		
Age (months)	**6**	**12**	**18**	**6**	**12**	**18**	**6**	**12**	**18**
BW (g)	455 ± 60	501 ± 11	511 ± 20	364 ± 70	437 ± 13	409 ± 23	413 ± 90	451 ± 70	446 ± 12
RVW (g)	0.29 ± 0.01	0.34 ± 0.06	0.31 ± 0.01	0.25 ± 0.02	0.40 ± 0.02	0.37 ± 0.06	0.26 ± 0.01	0.32 ± 0.01	0.39 ± 0.05
RVW/BW (g/kg)	0.63 ± 0.04	0.56 ± 0.02	0.61 ± 0.04	0.67 ± 0.04	0.90 ± 0.06	0.76 ± 0.07	0.64 ± 0.02	0.72 ± 0.03	0.88 ± 0.11
LVW/BW (g/kg)	2.20 ± 0.04	2.30 ± 0.06	2.51 ± 0.19	2.95 ± 0.07	3.73 ± 0.23*	3.73 ± 0.18*	2.90 ± 0.06	3.00 ± 0.05	3.56 ± 0.15*

Values are means ± SE; n = 6 rats in each group. BW, body weight; RVW, right ventricular weight; RVW/BW, right ventricular weight/body weight ratio; LVW/BW, left ventricular weight/body weight ratio. * $p < 0.05$ vs. 6 month old rats in the same strain.

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Fig. 5.1 Changes in systolic blood pressure (A) and left ventricular weight (B) in Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure-prone (SHHF) rats at 6, 12, 18 months of age. Values are means ± SE. n = 5-6 rats per group. * $p < 0.05$, † $p < 0.05$ versus age-matched SD rats, § $p < 0.05$ versus age-matched SD and SHHF rats.

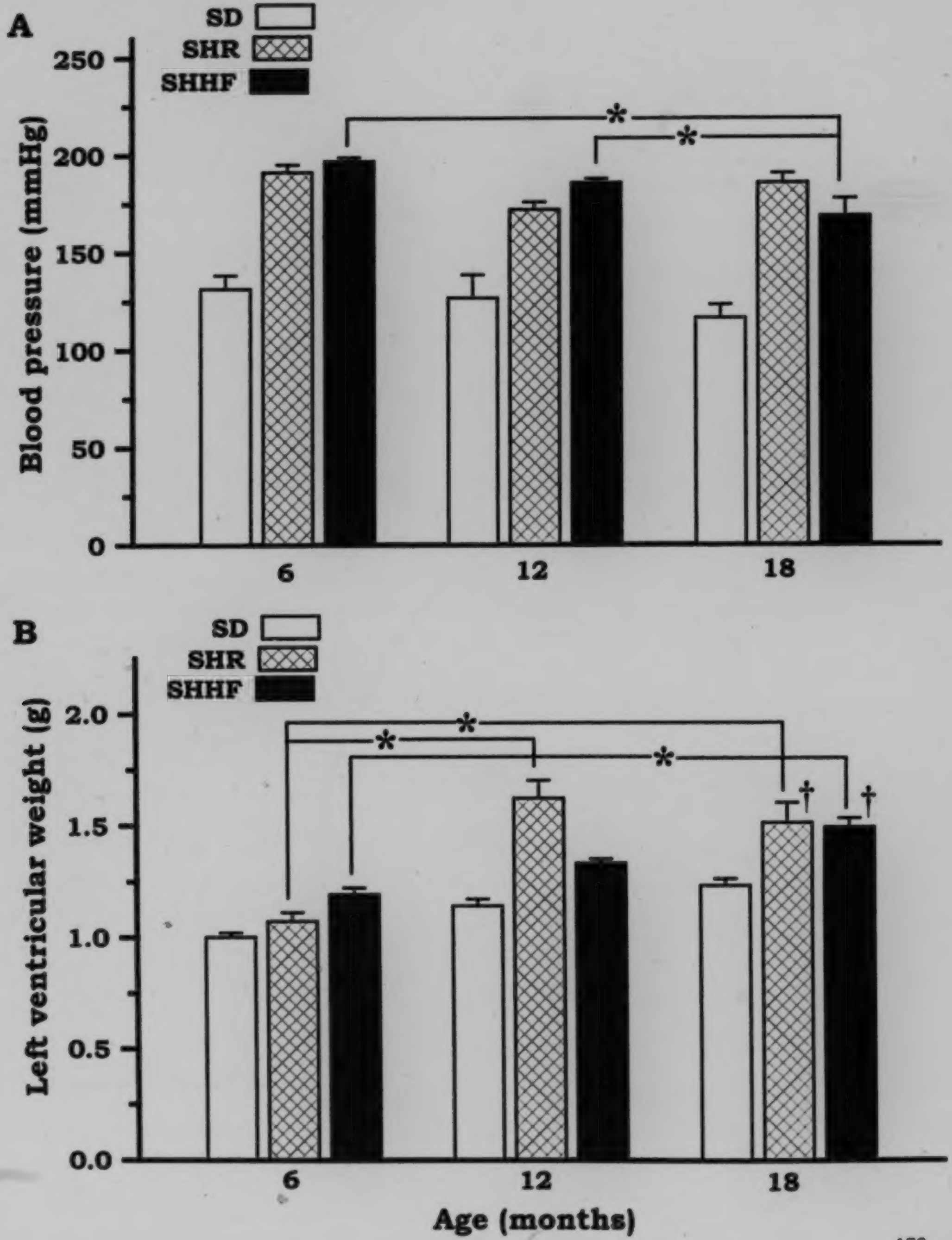


Fig. 5.2 Changes in plasma NO_x^- (A) and Ca^{2+}-dependent NOS activity (B) in Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure-prone (SHHF) rats at 6, 12 and 18 months of age. Values are means ± SE. n = 6 rats per group. * $p < 0.05$, † $p < 0.05$ versus age-matched SD rats, ‡ significant at $p < 0.05$ versus age-matched SHR rats.

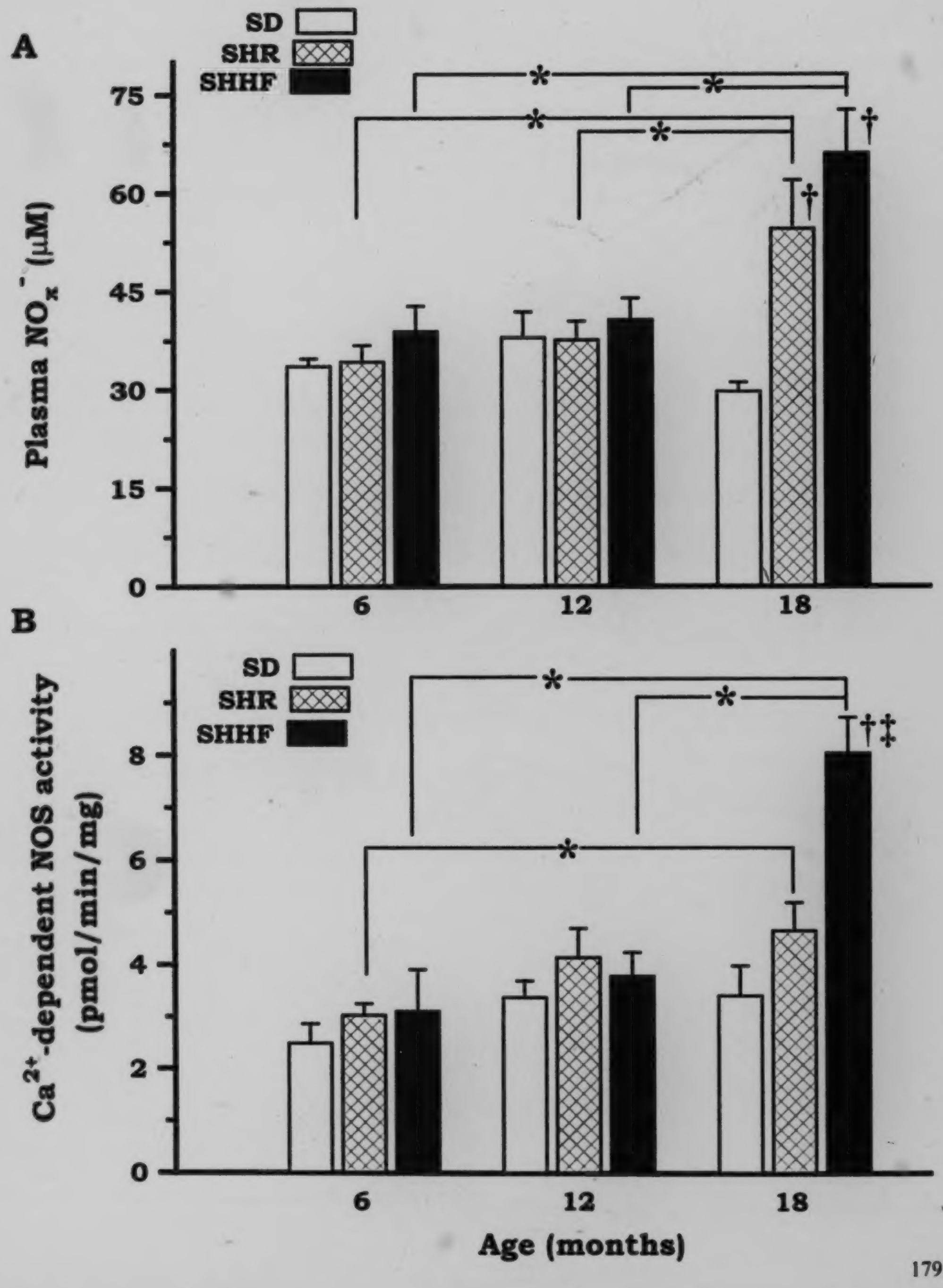


Fig. 5.3 Western blot and densitometric analysis of endothelial NOS (eNOS) in crude supernatant of left ventricular homogenates from Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure-prone (SHHF) rats at 6, 12 and 18 months of age. A) Western blot of eNOS expression in 18 month old SD, SHR and SHHF rats. 90 μg protein were loaded per lane. B) Densitometric analysis of changes in eNOS protein expression with age in the three strains of rats. Data are presented as a percent relative to the same external control run in each blot (heart from a 8 week old SD rat). Values are means ± SE. n = 5 rats per group. Mr: Molecular weight markers (kDa), * $p < 0.05$, † $p < 0.05$ versus age-matched SD or SHR rats.

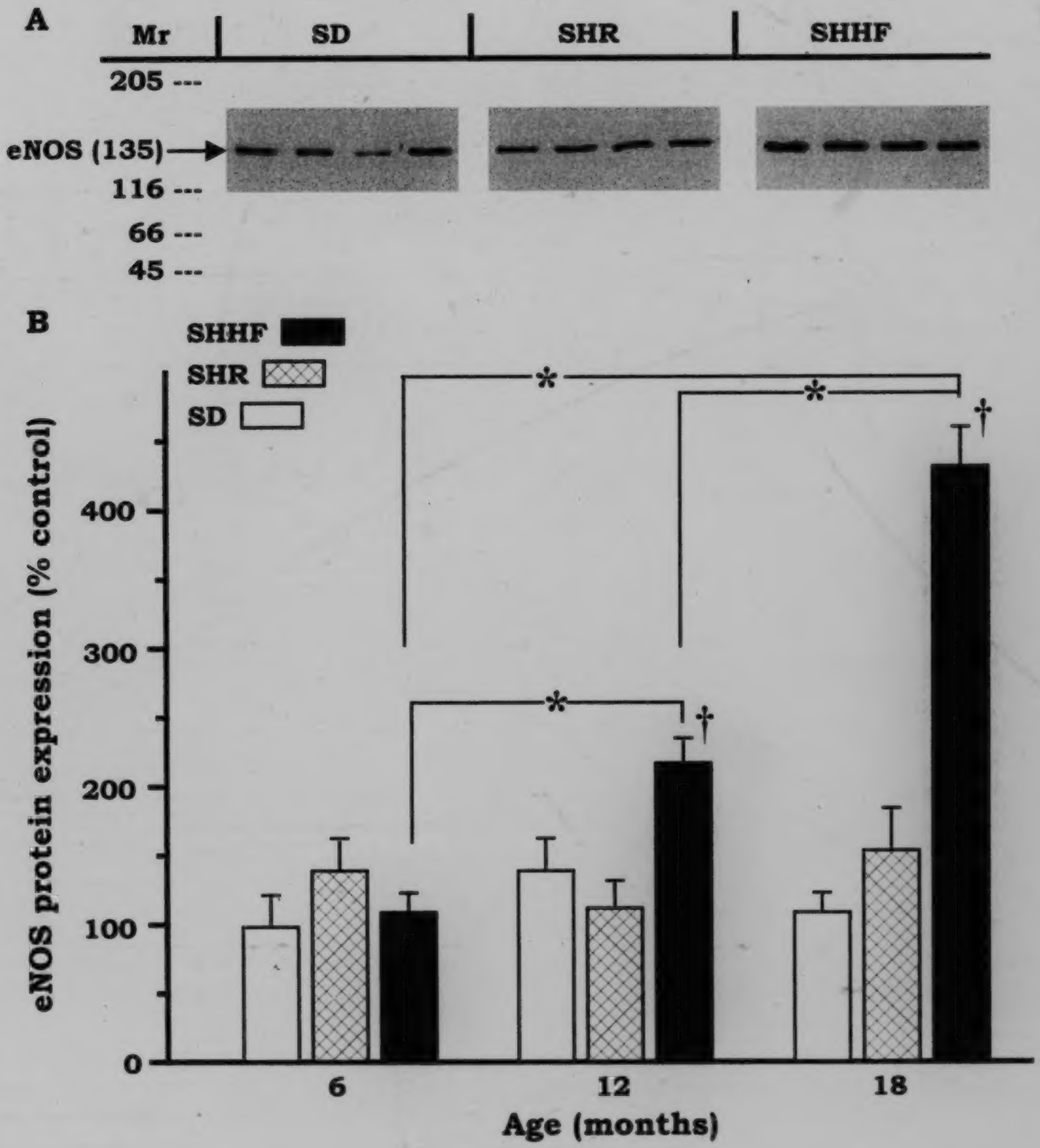
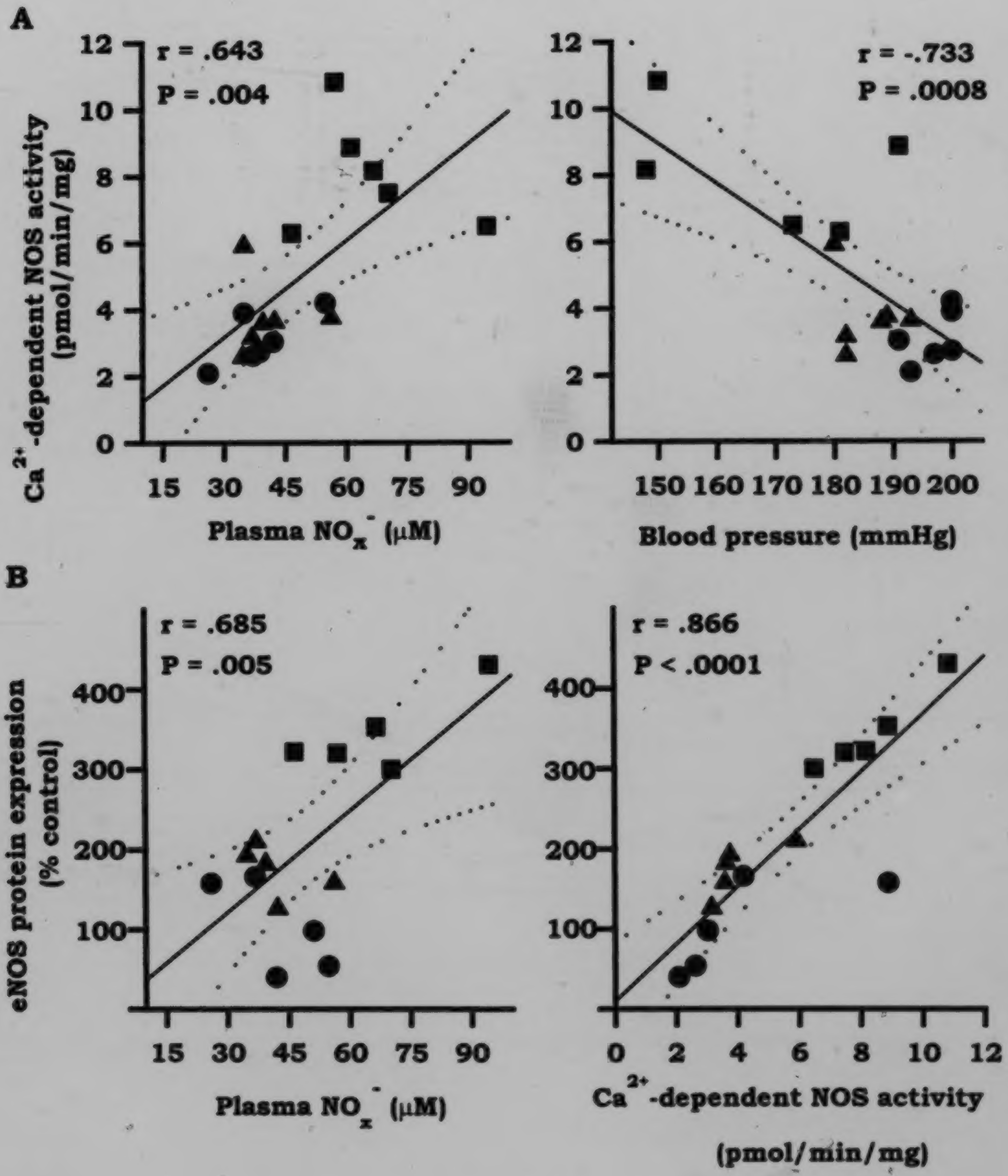


Fig. 5.4 Linear regression analysis of: A) ventricular Ca^{2+}-dependent NOS activity versus plasma NO_x^- (left panel, n = 18) or systolic blood pressure (right panel, n=17). B) ventricular eNOS protein expression versus plasma NO_x^- (left panel, n = 15) or cardiac Ca^{2+}-dependent NOS activity (right panel, n = 15) in SHHF rats. Dotted lines show 95% confidence interval. ●, 6-month-old rats; ▲, 12-month-old rats; and ■, 18-month-old rats.



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CHAPTER VI

CONCLUSIONS, LIMITATIONS & FUTURE DIRECTIONS

Exercise training and vascular reactivity

Previous studies have investigated exercise-induced changes in vascular reactivity including endothelium-mediated responses using isolated aortic segments (5, 6, 8, 9, 12). To confirm the *in vitro* data, the effect of swim exercise training on systemic vascular reactivity was investigated *in vivo*, by measuring the changes in MABP in response to the administration of endothelium-dependent and independent vasoconstrictor and dilator agents (Chapter II). Several limitations of this study warrant further discussion. Arterial blood pressure is determined by the cardiac output and systemic vascular resistance. Accordingly, changes in cardiac output caused by the administration of vasoactive drugs may have influenced MABP, thus possibly confounding the results obtained. Unfortunately, the heart rate was not recorded during these experiments, therefore the effect of these drugs on cardiac output cannot be substantiated. A better index for systemic vasoreactivity may be the peripheral vascular resistance. On the other hand, MABP is related to vascular tone in all peripheral organs and tissues and could be influenced by local changes in any vascular bed. The effect of altered tone through one vascular bed may be abrogated or augmented by opposite or similar changes in another bed. Aside from that, measurements of MABP were performed in anesthetized rats and I cannot eliminate a possible effect of anesthesia on MABP.

The effects of bradykinin (endothelium-dependent vasodilator) and SNAP (NO donor) on MABP, HQBF and HQC were studied at only two doses, both of which appeared to give maximal changes (Chapters II & III). A full dose-response curve is needed to elucidate their complete action and confirm the findings. As the vasoreactivity of the superior mesenteric bed was studied *in* *vitro*, a more accurate evaluation of the changes induced by exercise training in this bed may be obtained if experiments had been performed *in vivo*.

Exercise training and nitric oxide synthases

Our data from swim trained rats indicates that eNOS activity and protein expression were upregulated in the aorta, lung and atria (Chapters II). Whereas the changes in aortic and pulmonary eNOS may be readily explained by the increased blood flow and shear stress on the vascular endothelium leading to enhanced NO production (4, 18, 27) and eNOS expression (19, 22, 31), I can only speculate about the mechanism(s) causing these changes in atrial eNOS. Dilatation of the atria (increased stretching) (2, 20) due to enhanced venous return to the heart is one possible explanation. On the other hand, it is unlikely that hypertrophy of the atria *per se* contributed to the upregulation of eNOS since ventricular hypertrophy was not associated with similar changes in enzyme activity or expression. Similarly, to suggest that the lusitropic effect of NO may benefit diastolic filling and coronary perfusion in the setting of exercise (increased heart rate and coronary flow) is unlikely because ventricular NOS was unaltered.

How and why nNOS was upregulated in the hindquarters skeletal muscle of trained rats (Chapter III) is also of great interest. The localization of nNOS to the sarcolemma of skeletal muscle fibers near the motor endplate (15, 17) and the report by Ribera *et al.* that NO can modulate cholinergic neurotransmission (26) suggest that increased nNOS activity and protein in the trained rats may be related to the chronic increase in skeletal muscle activity and neuromuscular transmission. This is supported by the data of Reiser *et al.*

who found that chronic electrical stimulation of skeletal muscle enhances nNOS expression (25). Increased NO production, via nNOS, can be a physiological mechanism to limit O_2 consumption in actively contracting skeletal muscle since NO was shown to reversibly inhibit O_2 consumption and mitochondrial respiration in isolated cardiac muscle (35), or to enhance blood flow to the exercising muscle. Finally, because muscle metabolism is sensitive to NO at several sites (24), upregulation of nNOS may be an adaptive response of muscle metabolism to exercise training. The administration of a selective nNOS inhibitor (7-NINA or TFPI) into functionally isolated hindquarters of trained animals may help to clarify the role of nNOS in skeletal muscle. Alternatively, nNOS knockout animals may be used to examine the role of NO in skeletal muscle of trained animals. To strengthen the conclusion that visceral NOS activities are not altered by training, other abdominal organs and tissues (intestine, spleen, pancreas and mesentery) must be investigated in addition to the liver and kidney.

These studies investigated the alterations in NO pathway as part of the cardiovascular adaptations to swim exercise training and the results indicate that vascular, atrial and skeletal muscle NO formation are upregulated in trained rats. To complement these data, the effect of exercise training on other vasodilatory factors such as prostacyclin and EDHF should also be studied. Koller *et al.* in 1995 found that both NO and prostacyclin contribute to a similar extent (40-45% each) to flow-dependent arteriolar vasodilatation in skeletal muscle of exercised rats (16). Studies that look at exercise-induced changes in more than one vasoactive factor, including vasoconstrictors, clearly provide more comprehensive knowledge. Furthermore, the changes in cardiopulmonary NOS caused by swim training may be investigated using an eNOS knockout model of experimental animals.

Plasma NO_x^- in exercise and congestive heart failure

The use of plasma NO_x^- as an index of NO synthase activity *in vivo* requires knowledge of the confounding influence of nitrate from sources other than endogenous NOS activity. The plasma nitrate level is markedly affected by dietary intake of nitrate and nitrate-containing medications but not by cigarette smoking or nitrate formed by gastrointestinal microorganisms (14). Therefore, it has been proposed that dietary restriction is an absolute requirement if plasma NO_x^- is to be used as an index of NO formation *in vivo*. Furthermore, nitrate is widely distributed in the body (volume of distribution is 20 L in man), slowly cleared via the kidneys, and has a plasma half-life of 3.8 hours in dogs (37) and 7-8 hours in humans (14). Accordingly, a minimum of 1-2 days of nitrate restriction are needed before blood sampling for measurement of NO_x^- levels (assuming 6 half-lives are required to clear 98% of exogenous nitrate) (14). In our studies, rats (Chapters II, III & V) and dogs (Chapter IV) were fed commercially available food and drank tap water and no dietary restrictions were practiced. Plasma NO_x^- data may indeed be different if exogenous sources of nitrate were eliminated. Results may be different also if blood samples were taken immediately after the last bout of exercise (Chapters II & III). Since nitrate is excreted primarily via the kidneys (21, 33), the need to measure renal function concomitantly with plasma NO_x^- level cannot be overemphasized. The level of plasma NO_x^- does not allow any conclusion as to the source of NO production.

In addition to nitrate formation, NO liberated into the blood may react with specific protein thiols (including hemoglobin and albumin) to form *S*-nitrosothiols (29) or with free thiols such as glutathione to form nitrosoglutathione (7). Although protein nitrosothiols appear in human plasma at a moderate concentration of ~7 μM, it has been proposed that these nitrosothiols may serve as reservoirs for NO since their plasma concentration does not reflect their biological activity (13). It may also be assumed that these NO adducts or metabolites exist in the blood in equilibrium with each other. Future studies should investigate the physiological importance of these metabolites and their possible interaction with the nitrate pool in plasma.

Cardiac nitric oxide synthases in congestive heart failure

The changes in cardiac NOS activity and expression were examined in two different models of CHF, a rapid pacing model of CHF in dogs (Chapter IV) and a genetic rat model of spontaneous hypertension and heart failure (Chapter V). Although pacing-induced CHF in dogs may not model human heart failure secondary to ischemic or valvular heart diseases or inflammatory cardiomyopathies, it does mimic ventricular dysfunction in patients with chronic tachycardia. On the other hand, the SHHF rat model of CHF mimics many of the findings in human patients with heart failure caused by uncontrolled hypertension and diabetic cardiomyopathy. Our results indicate that the alterations in NOS activity and expression are not identical in these models. Further studies using other experimental models of CHF, e.g. myocardial infarction from coronary ligation or occlusion, or CHF induced by myocardial toxins are needed to uncover the changes in cardiac NOS which may be specific to each model.

Previous studies investigating the changes in cardiac NOS in humans with CHF reported an alteration in the protein and/or mRNA of iNOS (11, 30). More recent studies by Fuchuki and colleagues and Vejlstrup and co-workers reported data about enhanced expression of iNOS and eNOS in the failing human heart (10, 32). As changes in NOS protein and/or mRNA are not necessarily translated into parallel alterations in functional activity, we designed our studies to include measurements of the enzyme activity. In fact, NOS activities were first determined as Ca^{2+}-dependent or -independent and then Western blot analysis was performed when a significant change in the Ca^{2+}-dependent component was found as an indication of one of two specific enzymes responsible (eNOS or nNOS). Since Ca^{2+}-independent NOS activity was not found under any circumstances, Western blot analysis for iNOS was not performed. Ca^{2+}-dependent NOS activity positively correlated with eNOS protein expression in failing hearts, though to a variable degree. Similarly, there was some discrepancy between changes in Ca^{2+}-dependent NOS activity and those in eNOS protein level as determined in the lung, aorta and atria of sedentary and trained rats. There can be several reasons as to why certain amount of protein produces different levels of enzymatic activity in the same or different tissues: 1) post-translational modifications of eNOS, for example tyrosine phosphorylation or glycosylation, 2) the subcellular localization of eNOS in a particular tissue (caveolae vs. cytosolic) and 3) the presence of endogenous inhibitors of the enzyme.

The cellular or subcellular source of altered NOS in the heart or other tissues was not identified. This is important to understand the mechanism of the changes in NOS expression and to identify the functional consequences of these changes given the multiplicity of NOS isoforms, their divergent tissue distribution and the potential effects for NO on the myocardium and other tissues and organs. Our data therefore should be supplemented by immunohistochemical localization of NOS in the different tissues.

To substantiate the data of iNOS expression from studies in human failing hearts (10, 11, 30, 32), animals with end-stage heart failure of a comparable severity to that of humans should be investigated. Conversely, early changes in cardiac NOS in humans with heart failure have not been reported to date and the chamber specificity of such changes is unknown. Further studies should test the hypothesis that changes in eNOS occur early during the development of CHF, probably related to the cause or hemodynamic changes that occur in this setting and that iNOS is induced in the later stages of CHF regardless of the underlying etiology.

My results indicate that an increase in cardiac NO generation in the canine pacing model (Chapter IV) and the rat genetic model (Chapter V) of CHF occur via upregulation of eNOS. This is in contrast to the findings of previous studies of increased iNOS in human heart failure (10, 11, 30, 32). To add to these divergent findings, a recent study from Hintze's group reported a fall in cardiac NO production with the development of CHF in paced dogs (32). Since the amount of NO and not the level of NO by-products in plasma or the enzyme isotype generating it determine the degree to which cardiac function and/or metabolism may be affected, direct measurements of myocardial NO formation using NO probes or sensors may provide more accurate information about NO production in the failing heart. Whether NO is generated by the constitutive or inducible NOS, or by the myocardium itself or the coronary and endocardial endothelia may be of relative importance considering the well-documented paracrine effects of endothelial NO on the myocardium (3, 28, 34).

Nitric oxide and myocardial dysfunction in congestive heart failure

Given the potential effects of NO on cardiac muscle, recent studies have investigated the effect of altered NO production in the failing heart. Using NOS inhibitors, Yamamoto *et al.* found that increased NO production in failing myocytes attenuates the positive inotropic response to isoproterenol but does not alter baseline contractility of myocytes isolated from hearts of dogs with pacing-induced CHF (36). In a similar model of CHF, Xie *et al.* found an increase in O_2 consumption in isolated segments of failing canine hearts. Myocardial O_2 consumption falls in response to exogenous NO but not to bradykinin or carbachol suggesting diminished microvascular endothelial NO production in the failing heart (34). A recent study by the same group confirmed that cardiac NO generation is diminished in dogs with CHF and was found to be associated with increased glucose uptake and decreased fatty acid uptake. These changes in cardiac energy substrate metabolism were similar but not identical to those seen in normal dogs that received an inhibitor of NOS (23), suggesting that cardiac NO production may regulate substrate utilization and cellular respiration in the failing myocardium.

Recent studies, therefore, support both an up- and down-regulation of NO production in the failing canine heart. Furthermore, they indicate that altered NO synthesis in CHF may be detrimental by virtue of its negative inotropic properties (36) or beneficial through its vasodilatory (1) and antioxidant properties or its effects on cellular respiration and metabolism (23, 34, 35). Possible interventions may include the use of NO donors or NOS inhibitors to investigate their effects on the development of heart failure and whether they may accelerate or delay the development of myocardial dysfunction.

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